# DIFFERENTIAL MODULATION OF T-TYPE VOLTAGE GATED CALCIUM CHANNELS BY G-PROTEIN COUPLED RECEPTORS 

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#### Abstract

T-type voltage-gated calcium $\left(\mathrm{Ca}^{2+}\right)$ channels play critical roles in controlling neuronal excitability, firing patterns, and synaptic plasticity, although the mechanisms and extent to which T-type $\mathrm{Ca}^{2+}$ channels are modulated by G-protein coupled receptors (GPCRs) remains largely unexplored. Investigations into T-type modulation within native neuronal systems have been complicated by the presence of multiple GPCR subtypes and a lack of pharmacological tools to separate currents generated by the three T-type isoforms; $\mathrm{Ca}_{\mathrm{v}} 3.1, \mathrm{Ca}_{\mathrm{v}} 3.2$, and $\mathrm{Ca}_{\mathrm{v}} 3.3$. We hypothesize that specific $\mathrm{Ca}_{\mathrm{v}} 3$ subtypes play unique roles in neuronal physiology due to their differential functional coupling to specific GPCRs.

Co-expression of T-type channel subtypes and GPCRs in a heterologous system allowed us to identify the specific interactions between muscarinic acetylcholine (mAChR) or metabotropic glutamate (mGluR) GPCRs and individual $\mathrm{Ca}_{\mathrm{v}} 3$ isoforms. Perforated patch recordings demonstrated that activation of $\mathrm{G} \alpha_{q / 11}$-coupled GPCRs had a strong inhibitory effect on Cav 3.3 T-type $\mathrm{Ca}^{2+}$ currents but either no effect or a stimulating effect on $\mathrm{Ca}_{\mathrm{v}} 3.1$ and $\mathrm{Ca}_{\mathrm{v}} 3.2$ peak current amplitudes. Further study of the inhibition of $\mathrm{Ca}_{v} 3.3$ channels by a specific $\mathrm{G} \alpha_{q / 11}$-coupled mAChR (M1) revealed that this reversible inhibition was associated with a concomitant increase in inactivation kinetics. Pharmacological and genetic experiments indicated that the M1 receptor-mediated inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.3$ occurs specifically through a $\mathrm{Ga}_{\mathrm{q} / 11}$ signaling pathway that interacts with two distinct regions of the $\mathrm{Ca}_{\mathrm{v}} 3.3$ channel.

As hypothesized, the potentiation of $\mathrm{Ca}_{\mathrm{v}} 3.1$ channels by a $\mathrm{G}_{\mathrm{q} / 11}-$ coupled mGluR (mGluR1) initially characterized in the heterologous system was also observed in a native neuronal system: the cerebellar Purkinje cell (PC). In recordings on PCs within acute cerebellar slices, we demonstrated that the potentiation of $\mathrm{Ca}_{\mathrm{v}} 3.1$ currents by mGluR1 activation is strongest near the threshold of T-type currents, enhancing the excitability of PCs. Ultrafast two-photon $\mathrm{Ca}^{2+}$ imaging demonstrated that the functional coupling between mGluR1 and T-type transients occurs within dendritic spines, where synaptic integration and plasticity occurs. A subset of these experiments utilized physiological synaptic activation and specific mGluR1 antagonists in wild-type and $\mathrm{Ca}_{\mathrm{v}} 3.1$ knock-out mice to show that the mGluR1-mediated potentiation of $\mathrm{Ca}_{\mathrm{v}} 3.1$ T-type currents may promote synapse-specific $\mathrm{Ca}^{2+}$ signaling in response to bursts of excitatory inputs.


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## LIST OF ABBREVIATIONS

ACSF: artificial cerebrospinal fluid
AMPA: $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AOD: acousto-optic deflectors
AP: action potential
$\mathrm{Ba}^{2+}$ : barium
BBS: bicarbonate-buffered saline
$\mathrm{Ca}^{2+}$ : calcium
CAMKII: calcium/calmodulin kinase II
CCh: carbachol
$\mathrm{Cd}^{2+}$ : cadmium
CF: climbing fiber
CNS: central nervous system
CPA: cyclopiazonic acid
CRFR: corticotrophin-releasing factor receptor
DAG: diacylglycerol
DCN: deep cerebellar nuclei
DRG: dorsal root ganglion
EPSP: excitatory postsynaptic potential
$\mathrm{Ga}_{t}$ : transducin
GAERS: genetic absence epilepsy rat from Strasbourg
GC: granule cell
GDP- $\beta$-S: guanosine-5'-O-(2-thiodiphosphate)
GPCR: G-protein coupled receptor
HEK: human embryonic kidney
HVA: high voltage-activated
IGE: idiopathic generalized epilepsy
Ih: hyperpolarization-activated inward current
$\mathrm{I}_{\mathrm{L}}$ : leak current
$\mathrm{IP}_{3}$ : inositol-1,4,5-trisphosphate
$\mathrm{IP}_{3} \mathrm{R}: \quad \mathrm{IP}_{3}$ receptor
$\mathrm{K}^{+}$: potassium
KO: knock-out
LTD: long term depression

LTP: long term potentiation
LVA: low voltage-activated
mAChR : muscarinic acetylcholine receptor
MAPK: mitogen-activated protein kinase
MF: mossy fiber
mGluR: metabotropic glutamate receptor
$\mathrm{Na}^{+}$: sodium
$\mathrm{Ni}^{2+}$ : nickel
NMDA: N-methyl D-aspartate
nRT: thalamic reticular neuron
palpeptide: N-terminal palmitoylated decapeptide
PC: Purkinje cell
PF: parallel fiber
$\mathrm{PIP}_{2}$ : phosphatidylinositol-4,5-bisphosphonate
PKA: protein kinase A
PKC: protein kinase C
PLC: phospholipase C
PMA: phorbol 12-myristate 13-acetate
PNS: peripheral nervous system
POIs: points of interest
PTK: protein tyrosine kinase
PTX: pertussis toxin
RGS: regulator of G-protein signaling
$\mathrm{R}_{\mathrm{N}}$ : input resistance
$\mathrm{R}_{\mathrm{S}}$ : series resistance
SCG: superior cervical ganglion
sEPSC: slow excitatory postsynaptic current
sER: smooth endoplasmic reticulum
SWD: spike-and-wave discharge
TC: thalamocortical relay neuron
TRPC: transient receptor potential channel
UCN: urocortin
wt: wild-type

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## DEDICATION

To my saviour, Jesus Christ my loving wife, Sara and my precious son, Joshua

## CO-AUTHORSHIP STATEMENT

Along with input and feedback from other researchers, I designed and performed all of the experiments and analyzed the experimental results for all experiments, except for those shown in Figure 3.2E,F; Figure 3.6; and Figure 3.7. In other words, I performed all experiments except for the electrophysiological recordings on PCs that involved $\mathrm{Ca}^{2+}$ imaging or wild-type, $\mathrm{Ca}_{\mathrm{v}} 2.3 \mathrm{KO}$ and $\mathrm{Ca}_{\mathrm{v}} 3.1$ KO mice. As stated in the section 2.4.1 of Chapter 2, I also did not generate the $\mathrm{Ca}_{\mathrm{v}} 3.1-\mathrm{Ca}_{\mathrm{v}} 3.3$ chimeric channels used in Figure 2.7 or any other channel-plasmid constructs.

In terms of manuscript preparation, I wrote the entire manuscript for Chapter 2, with subsequent editing from Terry Snutch and other authors. For Chapter 3, I wrote the first draft for all sections except for section 3.3 and parts of sections 3.2 and 3.4 (written by Philippe Isope and Stephane Dieudonne). I offered editing and feedback on the sections that I did not write for Chapter 3.

I designed and wrote the entire review article shown in Appendix 1, with subsequent editing by Terry Snutch. For the research article in Appendix 2, I helped plan and design several critical experiments, I performed the experiments on the modulation of all three human T-type channels by UCN as well as the recovery from inactivation experiments, and I helped edit the paper and wrote a small section of the discussion.

## 1 INTRODUCTION

### 1.1 Voltage-gated calcium channel overview

### 1.1.1 Historical context

With more than 39,000 publications currently listed in PubMed, the field of voltage-gated calcium $\left(\mathrm{Ca}^{2+}\right)$ channel research has developed exponentially from its inception over 50 years ago through detailed characterizations in native systems and to the present molecular structure-function analyses of multiple channel classes. A recurrent theme throughout this rapidly-expanding trajectory has been that the selective modulation of $\mathrm{Ca}^{2+}$ channel activity by various cellular signaling pathways contributes to a wide variety of physiological functions.

The first evidence concerning the existence of specific $\mathrm{Ca}^{2+}$ channel-mediated activity emerged in the 1950s. In the era when Hodgkin and Huxley were characterizing the sodium $\left(\mathrm{Na}^{+}\right)$-driven action potentials (APs) in the squid giant axon, Bernard Katz, Paul Fatt, and Bernard Ginsborg discovered a novel form of electrical excitability in the large muscle cells of crab and crayfish that was independent of $\mathrm{Na}^{+}$gradients and appeared to involve $\mathrm{Ca}^{2+}$ spikes (Fatt and Ginsborg, 1958; Fatt and Katz, 1953). As with many novel and unexpected scientific discoveries, initial progress and acceptance was slow. Two key contributors in this "incubation period" of $\mathrm{Ca}^{2+}$ channel discovery were Harald Reuter and Albrecht Fleckenstein. Reuter performed voltage-clamp recordings on Purkinje fibers of the heart to directly demonstrate a transmembrane $\mathrm{Ca}^{2+}$ current linked to excitation-contraction coupling (Reuter, 1967) while Fleckenstein discovered that certain small organic compounds including verapamil and nifedipine (a dihydropyridine) blocked the $\mathrm{Ca}^{2+}$ signaling relating to excitation-contraction coupling (reviewed in (Fleckenstein, 1983)). The dihydropyridines were later shown to be antagonists of voltage-gated $\mathrm{Ca}^{2+}$ channels and proved crucial in the purification and characterization of $\mathrm{Ca}^{2+}$ channels.

In the late 1970s, voltage-clamp studies in many large invertebrate preparations started to open up the $\mathrm{Ca}^{2+}$ channel field. Building on the $\mathrm{Ca}^{2+}$ hypothesis of synaptic transmission by Katz and Miledi (Katz and Miledi, 1970), Rodolfo Llinas and colleagues used recordings on the squid stellate ganglion to reveal presynaptic, inward $\mathrm{Ca}^{2+}$ channel currents that were directly linked to postsynaptic potentials, and thus, neurotransmitter release (Llinas et al., 1976). Llinas went on to apply these findings to mammalian neuronal systems, where he characterized the electrophysiological properties and $\mathrm{Ca}^{2+}$ channel activity in cells such as the cerebellar Purkinje cell (PC) (Llinas and Sugimori, 1980a, b). Concomitantly, Susuma Hagiwara characterized $\mathrm{Ca}^{2+}$ conductances from various invertebrate tissues ranging from the egg cell membrane of starfish to barnacle giant muscle fibers (Hagiwara et al., 1975). Together, these studies provided the first evidence for the existence of more than one type of voltage-gated $\mathrm{Ca}^{2+}$ channel.

The proliferation of patch clamp recording technology in the 1980s enabled the $\mathrm{Ca}^{2+}$ channel field to explode, as single-cell voltage-clamp recordings were now possible on various types of neurons
throughout the brain, spinal cord, and peripheral nervous system (PNS). Building on Hagiwara's work, Carbone and Lux, along with Llinas and others, divided $\mathrm{Ca}^{2+}$ channel currents into two groupings, low voltage-activated (LVA) and high voltage-activated (HVA) conductances, based on the level of depolarization required to first activate the channels (Carbone and Lux, 1984). Subsequent extensive work in various native systems by Michael Adams, Clay Armstrong, Kurt Beam, Bruce Bean, Peter Hess, Ed McCleskey, Baldamero Olivera, and Richard Tsien led to the identification of multiple $\mathrm{Ca}^{2+}$ channel classes based on biophysical differences and differential sensitivities to pharmacological tools and venom toxins (reviewed in (Tsien and Barrett, 2005)). The $\mathrm{Ca}^{2+}$ channel classes included L-type channels that were long lasting and had a large unitary conductance, N -type channels recorded from neurons and that were non L-type, P (and Q)-type channels that were described from cerebellar Purkinje cells, R-type channels that were toxin-resistant, and T-type channels that formed tiny and transient currents. T-type channels were thought to exclusively contribute to LVA currents, while all other classes contributed to HVA currents (Fig. 1.1).

The understanding of the molecular components underlying native $\mathrm{Ca}^{2+}$ channel currents began with the biochemical purification of the skeletal muscle (L-type) $\mathrm{Ca}^{2+}$ channel complex by Bill Catterall, Kevin Campbell, Franz Hoffman and others. Channel purification revealed that functional L-type $\mathrm{Ca}^{2+}$ channels contained multiple subunits including a pore-forming $\alpha_{1}$ subunit (based on dihydropyridine pore blockers binding to this subunit), and was followed by the cloning of the pore-forming $\alpha_{1 S}$ (now $\mathrm{Ca}_{\mathrm{v}} 1.1$ ) channel subunit by a team led by Shosaku Numa (reviewed in (Dolphin, 2006)). Terry Snutch subsequently showed that distinct from skeletal muscle, the nervous system expressed a family of $\mathrm{Ca}^{2+}$ channel $\alpha_{1}$ subunits and he was able to identify and clone neuronal L-type channels ( $\mathrm{Ca}_{\mathrm{v}} 1.2 / \alpha_{1 \mathrm{C}}$; $\mathrm{Ca}_{\mathrm{v}} 1.3 / \alpha_{1 \mathrm{D}}$ ), the $\mathrm{Ca}_{\mathrm{v}} 2.1 / \alpha_{1 \mathrm{~A}}$ channel that underlies P/Q-type currents, the $\mathrm{Ca}_{\mathrm{v}} 2.2 / \alpha_{1 \mathrm{~B}}$ channel that underlies N-type currents, and the $\mathrm{Ca}_{\mathrm{v}} 2.3 / \alpha_{1 \mathrm{E}}$ channel that partially underlies R-type currents (Snutch et al., 1990; Soong et al., 1993). The final chapter in the $\mathrm{Ca}^{2+}$ channel molecular cloning effort was achieved by in silico alignment searches of the C. elegans, rat and human genomes and led to the identification of three mammalian T-type channels $\left(\mathrm{Ca}_{\mathrm{v}} 3.1 / \alpha_{1 \mathrm{G}} ; \mathrm{Ca}_{\mathrm{v}} 3.2 / \alpha_{1 \mathrm{H}} ; \mathrm{Ca} 3.3 / \alpha_{11}\right)$ as well as an additional L-type channel ( $\mathrm{Ca}_{\mathrm{v}} 1.4 / \alpha_{1 \mathrm{~F}}$ ) (Cribbs et al., 1998; Lee et al., 1999b; McRory et al., 2004; McRory et al., 2001; Perez-Reyes et al., 1998) (reviewed in (Snutch et al., 2005)). Figure 1.1 summarizes the primary sequence relationships between all known $\mathrm{Ca}^{2+}$ channel $\alpha_{1}$ subunits and their corresponding historical native classifications.


Figure 1.1 - Calcium channel classifications and phylogeny.
Phylogenetic relationships between primary sequences of cloned $\mathrm{Ca}^{2+}$ channels. $\mathrm{Ca}_{\mathrm{v}} \alpha_{1}$ subunits are shown in black, with historical channel names in parentheses. Only membrane spanning segments and pore-forming loops are compared in this phylogeny. Calcium channel classes belonging to the HVA historical grouping are shown in magenta while $\mathrm{Ca}^{2+}$ channel classes belong to the LVA historical grouping are shown in blue.

### 1.1.2 General calcium channel biochemical composition

Functional HVA $\mathrm{Ca}^{2+}$ channels appear to be comprised of a multi-subunit complex containing a large ( 190 to 250 kDa ) pore-forming $\alpha_{1}$ subunit, an intracellular $\beta$ subunit, a transmembrane, disulfidelinked $\alpha_{2} \delta$ subunit, and a $\gamma$ subunit. Although the $\alpha_{1}$ subunit is responsible for most channel biophysical and modulation properties, the auxiliary subunits ( $4 \beta$ subunit genes, four $\alpha_{2} \delta$ subunit genes, and eight $\gamma$ subunit genes cloned to date) increase channel diversity and functional specialization by altering the trafficking, voltage-dependence, and kinetics of specific channel complexes (Stea et al., 1994) (reviewed in (Arikkath and Campbell, 2003)). The importance of these auxiliary subunits is emphasized by the fact that spontaneous mutations within them can cause epileptic and ataxic phenotypes (reviewed in (Adams and Snutch, 2007)).

The determination of the amino acid composition of the $\mathrm{Ca}^{2+}$ channel $\alpha_{1}$ subunits revealed that they are members of a gene superfamily of transmembrane ion channel proteins that includes voltagegated potassium $\left(\mathrm{K}^{+}\right)$and $\mathrm{Na}^{+}$channels. Calcium channels are most closely related to $\mathrm{Na}^{+}$channel $\alpha$ subunits, with each being composed of four homologous domains each containing six hydrophobic transmembrane segments (termed S1 to S6) and a pore-forming loop between S5 and S6 segments (Fig. 1.2). The pore-forming loop is responsible for the specificity and permeation properties of the channel,
while regularly arranged positively charged amino acids in the S 4 transmembrane segments contribute to the voltage-sensitive gating of the channel. Due to their critical functions, the transmembrane segments and pore regions are well conserved across all $10 \mathrm{Ca}^{2+}$ channel $\alpha_{1}$ subunit genes (Fig. 1.1). Overall, amino acid sequences of $\alpha_{1}$ subunits are over $70 \%$ identical within a channel subfamily (e.g. - $\mathrm{Ca}_{\mathrm{v}} 1$ ), while less than $40 \%$ identical between channel subfamilies (e.g. - $\mathrm{Ca}_{\mathrm{v}} 1$ versus $\mathrm{Ca}_{\mathrm{v}} 2$ ) (reviewed in (Catterall et al., 2005)). Most divergence occurs within putative cytoplasmic regions of the channel, such as the domain II - domain III linker and the C-terminal tail, where there is also significant variation in size. Of all $\mathrm{Ca}^{2+}$ channel groupings, T-type channels have the least amount of sequence identity compared to the other classes. In fact, cloned T-type channels lack entire structural motifs that are conserved in all HVA Ca ${ }^{2+}$ channel classes, including the auxiliary $\beta$ subunit binding site in the domain I-II linker and an EF hand $\mathrm{Ca}^{2+}$ binding motif in the C-terminus. Thus, the typical HVA auxiliary subunits likely do not have an essential role in forming functional native T-type currents and, as discussed below, the modulation of T-type channels by second messenger-dependent pathways also differs from the HVA Ca ${ }^{2+}$ channels.


Figure 1.2 - Structural features of the $\alpha_{1}$ calcium channel subunit.
Common structural features of all four-domain $\mathrm{Ca}^{2+}$ channel $\alpha_{1}$ subunits include four homologous domains (brown labels) composed of six transmembrane segments (labeled 1 to 6 in domain I) and a pore forming loop (blue region) between segments 5 and 6 . The $4^{\text {th }}$ transmembrane segments (red) have regularly-spaced, positively charged amino acids and form the voltage sensor. The N-terminus, Cterminus and interdomain linkers are all intracellular (cytoplasmic).

### 1.1.3 Calcium channel classes

Original classifications divided $\mathrm{Ca}^{2+}$ channels into HVA and LVA subgroups (Fig. 1.1). T-type channels were defined as underlying LVA currents and could be distinguished based upon the small depolarizations required for channel opening and their small single channel conductance, slow deactivation kinetics and fast activation and inactivation kinetics. Conversely, all other $\mathrm{Ca}^{2+}$ channel subtypes formed HVA currents generally characterized by larger depolarizations required for opening, larger conductances, faster deactivation kinetics and variable kinetics and voltage-dependence of activation and inactivation. The HVA currents also possess a larger amplitude when extracellular $\mathrm{Ca}^{2+}$ is replaced with $\mathrm{Ba}^{2+}$, while permeability of $\mathrm{Ca}^{2+}$ versus $\mathrm{Ba}^{2+}$ through native LVA currents is more variable and often equal. However, the identification of "low-threshold" Ca ${ }^{1} 1.3$ L-type currents (reviewed in (Lipscombe et al., 2004)) and Ca, 2.3 currents (Bourinet et al., 1996b) has led to some confusion concerning the "low voltage-activated" classification. In this regard, "T-type" is the preferred term used when specifically describing the $\mathrm{Ca}_{\mathrm{v}} 3$ channel grouping. Figure 1.3 includes representative characteristics of a T-type channel ( $\mathrm{Ca}_{\mathrm{v}} 3.1$ ) versus a typical HVA Ca ${ }^{2+}$ channel ( $\mathrm{Ca}_{\mathrm{v}} 1.2$ ) to highlight some main biophysical differences between these two $\mathrm{Ca}^{2+}$ channel groupings.


Figure 1.3 - Representative differences between T-type and HVA calcium channels.
All properties, except for the single channel recording in B, were measured by the author from HEK cells transiently expressing a population of typical T-type channels ( $\mathrm{Ca}_{\mathrm{v}} 3.1$, blue) or typical HVA channels ( $\mathrm{Ca}_{\mathrm{v}} 1.2$, magenta) using whole-cell voltage clamp recordings at room temperature. A) T-type $\mathrm{Ca}^{2+}$ channels typically activate at much more hyperpolarized potentials than HVA Ca ${ }^{2+}$ channels. B) Cellattached, single channel recordings of native T-type (top) and L-type (bottom) currents in chick sensory neurons using $110 \mathrm{mM} \mathrm{Ba}^{2+}$. Voltage protocols (in mV ) are shown above sweeps of current traces. Ttype channels have a much smaller single channel conductance ( 8 pS ) than L-type currents ( $\sim 25 \mathrm{pS}$ ). Data adapted with permission from (Fox et al., 1987b). C-D) Voltage-clamp waveform protocol shown on top. C) T-type channels have faster activation and inactivation kinetics compared to HVA channels. D) After variable length test depolarizations to maximally open the channels, T-type channels have a slower rate of channel closing (deactivation) upon repolarization when compared to HVA channels.

The $\mathrm{Ca}_{\mathrm{v}} 1 \mathrm{Ca}^{2+}$ channel subfamily $\left(\mathrm{Ca}_{\mathrm{v}} 1.1\right.$ through $\left.\mathrm{Ca}_{\mathrm{v}} 1.4\right)$ encodes for the L-type class of $\mathrm{Ca}^{2+}$ channels. These channels are generally characterized by a large single channel conductance, minimal voltage-dependent inactivation and large $\mathrm{Ca}^{2+}$-dependent inactivation. L-type channels are predominantly expressed in skeletal, cardiac and smooth muscle where they are involved in excitationcontraction coupling and AP propagation (reviewed in (Flucher and Franzini-Armstrong, 1996)). In other cell types, the large window currents (membrane potentials where channels remain open at equilibrium) of L-type channels allows for tonic $\mathrm{Ca}^{2+}$ influx, providing a critical role in neurotransmitter release from photoreceptor and cochlear hair cells (McRory et al., 2004) (Appendix 3) and in hormone secretion from endocrine cells. In neurons, L-type channel expression appears to be mostly somatodendritic and its activity is linked to synaptic plasticity and changes in $\mathrm{Ca}^{2+}$-dependent gene expression (Hell et al., 1993) (reviewed in (Lipscombe et al., 2004)).

The $\mathrm{Ca}_{\mathrm{v}} 2 \mathrm{Ca}^{2+}$ channel subfamily is composed of the P/Q-type, N-type, and R-type classes. P/Qtype channels are expressed in the heart, pancreas, and pituitary as well as being widely distributed in neurons throughout the central nervous system (CNS) (Starr et al., 1991). In mammals, P/Q-type are the primary $\mathrm{Ca}^{2+}$ channel responsible for neurotransmitter release from the presynaptic terminals of both central and peripheral neurons (reviewed in (Catterall et al., 2005)). Native P-type and Q-type currents are distinguished based upon distinct kinetic, pharmacological, and modulatory properties and Snutch and colleagues demonstrated that alternative splicing of the $\mathrm{Ca}_{\mathrm{v}} 2.1$ gene generates both channel types (Bourinet et al., 1999).
$\mathrm{Ca}_{\mathrm{v}} 2.2$ channels make up native N -type currents: the only $\mathrm{Ca}^{2+}$ channel class with expression limited to the nervous system (Dubel et al., 1992). N-type currents are prominent in the presynaptic terminals, dendrites, and cell bodies of both central and sympathetic neurons, and play critical roles in the sensation and transmission of pain. New classes of drugs that specifically target N -type $\mathrm{Ca}^{2+}$ channels are currently being developed for clinical use in the treatment of chronic and neuropathic pain (reviewed in (Snutch, 2005)).
$\mathrm{Ca}_{\mathrm{v}} 2.3$ channels have several properties, such as ion selectivity and voltage-dependence of activation, that resemble native T-type currents (Bourinet et al., 1996b; Li et al., 2007; Soong et al., 1993). However, Cav 2.3 channels also exhibit some properties consistent with native high-threshold Rtype currents and there has been some contention in the literature as to how best classify this channel. $\mathrm{Ca}_{\mathrm{v}} 2.3$ channels are widely expressed in the heart, testes, pituitary, and central neurons and are functionally linked to neurotransmitter release, modulating rhythmic firing patterns, synaptic plasticity and neurosecretion (reviewed in (Catterall et al., 2005)).

The $\mathrm{Ca}_{\mathrm{v}} 3 \mathrm{Ca}^{2+}$ channel subfamily forms the T-type $\mathrm{Ca}^{2+}$ channel class and is composed of three separate genes: $\mathrm{Ca}_{\mathrm{v}} 3.1, \mathrm{Ca}_{\mathrm{v}} 3.2$, and $\mathrm{Ca}_{\mathrm{v}} 3.3$ (McRory et al., 2001). These subtypes/isoforms have a broad
expression pattern, including the ovaries, placenta, heart, kidney, smooth muscle, liver, adrenal cortex and neurons throughout the PNS and CNS. In many of these cell types, more than one T-type isoform is expressed. Non-neuronal functions of T-type channels include smooth muscle contraction, hormone secretion and cardiac pacemaker activity (reviewed in (Catterall et al., 2005)). The expression and functional roles of T-type channels are discussed in more detail in the following sections.

### 1.2 T-type voltage-gated calcium channels

### 1.2.1 T-type channel pharmacology

The study of native T-type currents has been hindered by two major issues: 1) some HVA channels actually activate at relatively negative potentials (e.g. $\mathrm{Ca}_{\mathrm{v}} 1.3$ and $\mathrm{Ca}_{\mathrm{v}} 2.3$ ), and 2 ) unlike the HVA Ca ${ }^{2+}$ channel classes, no high affinity specific channel antagonists have been discovered that clearly distinguish T-type currents from HVA currents or that distinguish between individual T-type subtypes. These issues were compounded in early investigations of native T-type currents, where biophysical properties and sensitivities to pharmacological antagonists such as nickel $\left(\mathrm{Ni}^{2+}\right)$ varied depending on the cell type. The recent cloning and characterization of three separate T-type isoforms has helped explain these divergent properties and has provided some clarification on the limitations and suitable uses of the pharmacological tools presently available. Although specific, high-affinity T-type blockers are lacking, the recent emergence of T-type channels as a potential novel therapeutic target for the treatment of pain, epilepsy, and hypertension (among other disorders) has led to a strong effort to synthesize specific antagonistic compounds. As an illustration, over a dozen reviews have been published just in the last two years that directly address the development of T-type-specific antagonists and/or the therapeutic potential of T-type channel blockade.

While one of the earliest T-type current antagonists to be identified was $\mathrm{Ni}^{2+}$, the sensitivity to this agent is highly variable between different native systems. For example, $\mathrm{Ni}^{2+}$ inhibits T-type currents in chick skeletal muscle cells with an $\mathrm{IC}_{50}$ of $21 \mu \mathrm{M}$ (Satoh et al., 1991), while it is a much less effective T-type inhibitor $\left(\mathrm{IC}_{50}=110 \mu \mathrm{M}\right)$ in cerebellar PCs (Kaneda et al., 1990). Molecular identification of the three $\mathrm{Ca}_{\mathrm{v}} 3$ channels revealed that $\mathrm{Ca}_{\mathrm{v}} 3.2$ is the only T-type isoform highly sensitive to $\mathrm{Ni}^{2+}$, with an $\mathrm{IC}_{50}$ of $12 \mu \mathrm{M}$ compared to $\mathrm{IC}_{50}$ 's of $250 \mu \mathrm{M}$ and $216 \mu \mathrm{M}$ for $\mathrm{Ca}_{\mathrm{v}} 3.1$ and $\mathrm{Ca}_{\mathrm{v}} 3.3$, respectively (Lee et al., 1999c). The use of $\mathrm{Ca}_{\mathrm{v}} 3.1-\mathrm{Ca}_{\mathrm{v}} 3.2$ chimeric channels and point-mutated channels demonstrated that the high affinity inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.2$ channels is due to a histidine residue in the S3-S4 loop of domain I that helps form $\mathrm{Ni}^{2+}$ binding pocket on the extracellular surface of the channel. Both $\mathrm{Ca}_{\mathrm{v}} 3.1$ and $\mathrm{Ca}_{\mathrm{v}} 3.3$ channels have a glutamine at this residue (Kang et al., 2006). Nickel blocks Cav 1.2 L-type and Cav 2.3 Rtype channels with a higher potency than either $\mathrm{Ca}_{\mathrm{v}} 3.1$ or $\mathrm{Ca}_{\mathrm{v}} 3.3$ T-type channels (Lee et al., 1999c; Zamponi et al., 1996). Therefore, low concentrations (e.g. - $50 \mu \mathrm{M}$ ) of $\mathrm{Ni}^{2+}$ can be used to selectively
block $\mathrm{Ca}_{\mathrm{v}} 3.2$ T-type currents (with minimal blockade of $\mathrm{Ca}_{\mathrm{v}} 3.1$ and $\mathrm{Ca}_{\mathrm{v}} 3.3$-mediated T-type currents), but $\mathrm{Ca}_{\mathrm{v}} 2.3$-mediated R-type currents will also be significantly attenuated at these concentrations.

Another polyvalent cation often used for studying T-type function is cadmium $\left(\mathrm{Cd}^{2+}\right)$. Low concentrations of $\mathrm{Cd}^{2+}(20$ to $100 \mu \mathrm{M})$ completely block all HVA Ca ${ }^{2+}$ current classes, while leaving Ttype currents relatively unaffected (Berrow et al., 1997; Fox et al., 1987a; Tai et al., 2006). Thus, application of $\mathrm{Cd}^{2+}$ can be used to study T-type currents in isolation from all other $\mathrm{Ca}^{2+}$ channels. The specificity of $\mathrm{Cd}^{2+}$ for high affinity HVA channel block is due to the four amino acid (EEEE) selectivity filter located in the four pore-forming loops of HVA channels. T-type channels have an EEDD sequence at this locus, and $\mathrm{Cd}^{2+}$ sensitivity is conferred to $\mathrm{Ca}_{\mathrm{v}} 3.1$ channels when mutated to either EEED or EEEE (Talavera et al., 2001).

Besides divalent cations, the search for selective T-type antagonists present in nature has thus far only revealed a single peptide toxin. Kurtoxin is a peptide purified from Parabuthus transvaalicus scorpion toxin and was initially described as a selective, high-affinity $\mathrm{Ca}_{\mathrm{v}} 3.1$ and $\mathrm{Ca}_{\mathrm{v}} 3.2$ blocker (Chuang et al., 1998). This peptide is also known to block voltage-gated $\mathrm{Na}^{+}$channels but not to inhibit recombinant HVA Ca ${ }^{2+}$ channels (Chuang et al., 1998). However, further studies within native thalamic and sympathetic neurons demonstrated that kurtoxin also inhibits N-type, R-type, and L-type currents with nanomolar affinities, thereby reducing its utility as a selective T-type antagonist (Sidach and Mintz, 2002).

There are a number of clinical agents that non-specifically target T-type channels. Mibefradil is the primary example of a therapeutic compound with efficacy in blocking T-type currents. This agent was in clinical use for treating hypertension and angina through its apparent block of T-type and L-type currents (Massie, 1997), but was later removed from the market for its potentially fatal inhibition of cytochrome enzymes essential for metabolizing other therapeutic compounds (reviewed in (Welker et al., 1998)). For research purposes, mibefradil was found to selectively inhibit T-type currents ( $\mathrm{IC}_{50}$ ranging from 14 nM to $1 \mu \mathrm{M}$ ) over HVA currents in some native systems, with state-dependent block causing greater inhibition of T-type currents at more depolarized potentials (McDonough and Bean, 1998). However, other studies showed that mibefradil can potently block R-type currents in the NG108-15 cell line (Randall and Tsien, 1997) and can also block N-type, L-type and P-type current at a concentration of $1 \mu \mathrm{M}$ in spinal motor neurons (Viana et al., 1997). Due to the non-specificity of mibefradil action, this compound is now deemed to be a non-specific T-type antagonist.

Some dihydropyridines can also block LVA currents at low micromolar concentrations in native systems, but sensitivity is highly variable and may be partly due to the presence of dihydropyridinesensitive $\mathrm{Ca}_{\mathrm{v}} 1.3$ currents that are low voltage-activated (Akaike et al., 1989) (reviewed in (Yunker, 2003)). However, dihydropyridines that are less effective at blocking L-type channels ( $\left.\mathrm{IC}_{50} \sim 40 \mu \mathrm{M}\right)$
have recently been shown to be reasonably potent T-type blockers $\left(\mathrm{IC}_{50} \sim 1 \mu \mathrm{M}\right)$. Novel specific T-type antagonists might potentially be generated from derivatives of these compounds (Kumar et al., 2002). A number of other therapeutic agents that have been shown to act on T-type channels at therapeuticallyrelevant levels, including diphenylbutylpiperidine neuroleptics (such as pimozide and penfluridol) (Santi et al., 2002), a phenylalkylamine antihypertensive (verapamil) (Freeze et al., 2006), the antidepressant trazadone (Kraus et al., 2007), succinimide antiepileptics (such as ethosuximide) (Gomora et al., 2001), volatile anesthetics (such as enflurane) (Joksovic et al., 2005), and the anesthetic/analgesic, nitrous oxide (Todorovic et al., 2001a). The blockade of T-type channels is likely only partly contributing to the physiological effects of these drugs, as they also act on a spectrum of molecular targets ranging from HVA Ca ${ }^{2+}$ channels to voltage-gated $\mathrm{Na}^{+}$and $\mathrm{K}^{+}$channels to G-protein coupled receptors (GPCRs) and ionotropic receptors. Thus, the use of these therapeutic agents as specific T-type antagonists is currently very limited. However, the efficacy of these agents in blocking T-type channels indicates that T-type channels could be important therapeutic targets for the treatment of conditions including epilepsy, pain, cardiac hypertrophy, ischemia, hypertension, cancer, and diabetes (reviewed in (Yunker, 2003)). In fact, since the discovery of mibefradil, many groups are attempting to design specific synthetic T-type blockers for therapeutic clinical use (Kim et al., 2007; Rhim et al., 2005).

### 1.2.2 T-type channel biophysical properties

T-type channels have many characteristic biophysical properties that are unique among voltagegated $\mathrm{Ca}^{2+}$ channels. These include a small single channel conductance, fast activation and inactivation kinetics, a relatively hyperpolarized voltage-dependence of activation and inactivation, and fast deactivation kinetics. The activation and inactivation kinetics of T-type channels are also strongly voltage-dependent. This creates a characteristic "crossing over" pattern in successively more depolarized traces of a conventional square-pulse current-voltage protocol. For neuronal T-type currents, the $\tau_{\text {inact }}$ can range from greater than 100 ms at activation thresholds (between -70 mV and -50 mV ) to less than 20 ms at maximal activating potentials ( $>-30 \mathrm{mV}$ ) (reviewed in (Huguenard, 1996)). Although T-type currents exhibit strong voltage-dependent inactivation, they do not inactivate in a $\mathrm{Ca}^{2+}$-dependent manner similar to HVA $\mathrm{Ca}^{2+}$ channels.

After initial cloning, the recombinant T-type isoforms were biophysically well characterized, and these properties have been thoroughly reviewed (Perez-Reyes, 2003). In brief, comparison of the three rat $\mathrm{Ca}_{\mathrm{v}} 3$ isoforms revealed that $\mathrm{Ca}_{\mathrm{v}} 3.1$ and $\mathrm{Ca}_{\mathrm{v}} 3.2$ have properties similar to "typical" native T-type currents, while $\mathrm{Ca}_{\mathrm{v}} 3.3$ possesses distinct biophysical properties. The $\mathrm{Ca}_{\mathrm{v}} 3.1$ and $\mathrm{Ca}_{\mathrm{v}} 3.2$ isoforms have fast activation and inactivation kinetics while $\mathrm{Ca}_{\mathrm{v}} 3.3$ channel activation and inactivation kinetics are much slower. The rat $\mathrm{Ca}_{\mathrm{v}} 3.3$ channels also have faster deactivation kinetics and a more hyperpolarized voltage dependence of activation and inactivation compared with the other two T-type isoforms (McRory
et al., 2001). All three isoforms have a characteristically fast recovery from inactivation, with the $\mathrm{Ca}_{\mathrm{v}} 3.1$ and $\mathrm{Ca}_{\mathrm{v}} 3.3$ channels recovering the fastest (Klockner et al., 1999). A recent paper comparing T-type biophysical parameters at room temperature and a physiological mammalian temperature $\left(37^{\circ} \mathrm{C}\right)$ demonstrated that increasing the recording temperature dramatically alters many of these properties in a non-linear, isoform-specific manner (Iftinca et al., 2006). In this regard, caution should be used when extrapolating specific T-type parameters measured at room temperature to models of physiological neuronal excitability.

Alternative splicing creates additional functional diversity in T-type channel activity and the study of these splice variants has provided insight into the roles of different structural regions in T-type gating properties. Alternative splicing in the human $\mathrm{Ca}_{\mathrm{v}} 3.1$ channel (Fig. 1.4) leads to several variants including: 1) insertion (exon 14, region $e$ ) in the domain II-III linker that shifts the voltage dependence of inactivation in the hyperpolarizing direction and also increases inactivation kinetics, 2) variation in exon 25 ( $a$ or $b$ forms) and 3) insertion of exon 26 (region $c$ ) in the domain III-IV linker that affects the voltage-dependence of activation and inactivation, and the kinetics and voltage-dependence of inactivation, respectively (Chemin et al., 2001a). The $\mathrm{Ca}_{\mathrm{v}} 3.1-a$ and $\mathrm{Ca}_{\mathrm{v}} 3.1-a e$ isoforms predominate in human brain regions such as the cerebellum and thalamus (Monteil et al., 2000). Additional alternative splicing has recently been identified in the Cav 3.1 C -terminus and can affect either current kinetics or the window current magnitude, depending upon the specific splice background (Emerick et al., 2006).

The human $\mathrm{Ca}_{\mathrm{v}} 3.3$ channel is alternatively spliced (Fig. 1.4) in the domain I-II linker, involving a deletion of exon 9 ( 35 amino acids), as well as in the carboxyl-terminus, involving a partial deletion of exon 33 (13 amino acids) (Mittman et al., 1999). These splicing events affect channel activation and inactivation kinetics interdependently, suggesting a possible direct interaction between the two channel regions (Murbartian et al., 2004). Overall, the above findings demonstrate that a majority of functional T-type splicing occurs in cytoplasmic channel regions and affects both the kinetics and voltagedependence of channel gating. The prevalence of cytoplasmic splicing could also be involved in differential sensitivities to intracellular second messenger modulation pathways, creating a mechanism for further channel specialization.


Figure 1.4 - Alternative splicing of intracellular exons in human $\mathrm{Ca}_{\mathrm{v}} 3$ channels.
Numbers correspond to exons that are alternatively spliced in $\mathrm{Ca}_{\mathrm{v}} 3.1$ (blue), $\mathrm{Ca}_{\mathrm{v}} 3.2$ (green), and $\mathrm{Ca}_{\mathrm{v}} 3.3$ (red) channels. Numbers in bold represent splicing that results in complete insertion or deletion of the exon, while numbers in italics represent exons that have partial deletions or insertions due to alternative splicing. The approximate locations of the alternatively spliced exons in the channel structure are indicated by arrows. Only alternative splicing events that occur in putative intracellular (cytoplasmic) regions and that result in functional channels are shown. Alternative splice variants were identified and characterized in (Chemin et al., 2001a; Emerick et al., 2006; Monteil et al., 2000; Murbartian et al., 2004; Zhong et al., 2006).

In addition to alternative splicing analyses, chimeric T-type channels and recombinant T-type channels with targeted deletions or point mutations have provided further insights into the biophysical structure-function relationships of $\mathrm{Ca}_{\mathrm{v}} 3$ channels. The $\mathrm{Ca}_{\mathrm{v}} 3$ domain I-II linker is highly divergent from that of HVA Ca ${ }^{2+}$ channels and has lost the ability to bind to $\beta$ ancillary subunits with high affinity. As hypothesized, replacement of the $\mathrm{Ca}_{\mathrm{v}} 3.1$ domain I-II linker with a $\mathrm{Ca}_{\mathrm{v}} 2.2$ domain I-II linker conferred some aspects of $\beta$-regulation to this T-type channel, but surprisingly causes the channel to activate at even lower voltages (Arias et al., 2005). The Cavv $3.1-\mathrm{Ca}_{\mathrm{v}} 2.2$ chimera also had greater functional expression than wild-type (wt) $\mathrm{Ca}_{\mathrm{v}} 3.1$ channels, leading to the hypothesis that the T-type domain I-II
linker may contain an ER retention signal. Experiments involving systematic deletion of regions of the domain I-II linker in $\mathrm{Ca}_{v} 3.2$ channels further support this hypothesis, as deleting the central region of the domain I-II linker increased T-type current density and surface expression (Vitko et al., 2007). Other deletions led to the postulation that the first 62 amino acids of the $\mathrm{Ca}_{\mathrm{v}} 3.2$ domain I-II linker are involved in regulating the voltage-dependence of activation and inactivation by acting as a gating particle that stabilizes the channel in the closed state (Vitko et al., 2007). Structural and electrophysiological experiments reveal that the proximal region of the domain I-II linker forms a putative helix-loop-helix structure that acts as a gating brake (Arias et al., 2008). Thus, the domain I-II linker appears to have a significant role in regulating channel expression and gating within T-type channels. It should be noted that the distal C -terminus of $\mathrm{Ca}_{\mathrm{v}} 3.3$ channels also affects channel expression and thus multiple intracellular regions are likely involved (Gomora et al., 2002).

Other regions of $\mathrm{Ca}_{\mathrm{v}} 3$ channels also appear to play a role in the voltage-dependence of T-type activation, as replacement of domains I, III, or IV in $\mathrm{Ca}_{\mathrm{v}} 3.1$ channels with wt $\mathrm{Ca}_{\mathrm{v}} 1.2$ sequence leads to HVA-like channel gating (an $\sim+40 \mathrm{mV}$ shift in $\mathrm{V}_{50 a c t}$ ) (Li et al., 2004). Surprisingly, sequence differences in the S 4 putative voltage sensor regions in these domains do not account for the large shifts in activation gating. However, mutations in the outermost positively charged arginine residues of the S4 transmembrane segment in domain IV of Cav 3.2 channels (Lam et al., 2005) and domains I, II, and III of $\mathrm{Ca}_{\mathrm{v}} 3.1$ channels (Kurejova et al., 2007) revealed that S 4 segments in T-type channels do, in fact, act as activation voltage-sensing domains. The selectivity filter EEDD locus in the four pore-forming loops of $\mathrm{Ca}_{\mathrm{v}} 3$ channels has also been shown to affect activation properties (Talavera et al., 2001), leading to the overall conclusion that multiple channel regions including the domain I-II linker, S4 segments, and poreforming loops all contribute to T-type activation gating.

The structural determinants of T-type inactivation have been more difficult to elucidate. High voltage-activated $\mathrm{Ca}^{2+}$ channels are proposed to inactivate by a mechanism similar to the voltage-gated $\mathrm{Na}^{+}$channel "hinged-lid" mechanism whereby the HVA domain I-II linker acts as a cytoplasmic inactivation particle that docks at a site formed by the S 6 segments of all four domains. In this model, other channel regions such as the C-terminus can regulate inactivation by altering the mobility of the inactivation particle (reviewed in (Stotz et al., 2004)). Consistent with this model, mutating domain III S6 residues that are well-conserved inactivation determinants in HVA channels shifted the voltagedependence of activation and altered the activation and inactivation kinetics of $\mathrm{Ca}_{\mathrm{v}} 3.1$ channels (Marksteiner et al., 2001). Furthermore, mutations in the S 6 segment of domain I in $\mathrm{Ca}_{\mathrm{v}} 3.1$ channels and deletion of the last 15 amino acids of the domain I-II linker in $\mathrm{Ca}_{\mathrm{v}} 3.2$ channels also slowed open channel inactivation (Arias et al., 2005; Vitko et al., 2005). Chimeric $\mathrm{Ca}_{\mathrm{v}} 3.1-\mathrm{Ca}_{\mathrm{v}} 1.2$ channel studies showed that a carboxyl region just after domain IV partly determines the fast inactivation of $\mathrm{Ca}_{\mathrm{v}} 3.1$ channels (Staes et al., 2001), suggesting a role for the C-terminus in T-type channel inactivation and supporting a model
similar to that of HVA channel inactivation. However, studies involving the slowly-inactivating $\mathrm{Ca}_{\mathrm{v}} 3.3$ channel appear to conflict with this model. Chimeric studies between $\mathrm{Ca}_{\mathrm{v}} 3.1$ and $\mathrm{Ca}_{\mathrm{v}} 3.3$ channels revealed that multiple structural elements contribute to the slow inactivation of $\mathrm{Ca}_{\mathrm{v}} 3.3$ currents, with inactivation kinetics becoming more " $\mathrm{Ca}_{\mathrm{v}} 3.3$-like" with increased $\mathrm{Ca}_{\mathrm{v}} 3.3$ sequence in the chimeric channels (Park et al., 2004). Furthermore, inserting the $\mathrm{Ca}_{\mathrm{v}} 3.1$ domain I-II linker into the $\mathrm{Ca}_{\mathrm{v}} 3.3$ channel had no significant effect on inactivation kinetics (Park et al., 2004). Another recent Cav 3.1- Cavv 3.3 chimeric study was also unable to determine specific structural regions of the $\mathrm{Ca}_{\mathrm{v}} 3.3$ channel directly responsible for its slower inactivation kinetics (Hamid et al., 2006).

One caveat in using chimeric and mutated channels to study the structural determinants of T-type inactivation is that macroscopic inactivation is coupled to activation in these channels. Increases in the closed-to-open transition rate can also increase the rate of macroscopic channel inactivation (Talavera and Nilius, 2006b). In fact, Talavera and Nilius show that there is a linear relationship between the activation and inactivation rates in many of the above noted T-type inactivation studies, suggesting that some of the identified structures may in fact be structural determinants of activation and not inactivation (Talavera and Nilius, 2006b). However, specific mutations in $\mathrm{Ca}_{\mathrm{v}} 3.1$ channels that affect activation but not inactivation kinetics demonstrate that macroscopic inactivation is not strictly linked to activation gating (Lam et al., 2005), and therefore, it is likely that activation and inactivation properties are determined by common T-type structural features. Overall, the understanding of structure-function relationships in T-type channels is incomplete, and currently "we still know very little about the mechanism underlying the basic functional features of T-type channels" (Talavera and Nilius, 2006a).

### 1.2.3 T-type channel expression

T-type channels are highly expressed in a variety of types of neurons throughout the CNS and PNS. Combining studies of T-type immunohistochemistry (Craig et al., 1999; McKay et al., 2006) with electrophysiological recordings of T-type currents (Isope and Murphy, 2005; Joksovic et al., 2005; Kavalali et al., 1997) reveals that $\mathrm{Ca}_{\mathrm{v}} 3$ channels and their functional currents are predominantly localized to the soma and dendrites of neurons, with the highest expression often occurring in dendritic regions. The functional significance of this subcellular localization will be further explored in the next section.

The first characterization of $\mathrm{Ca}_{\mathrm{v}} 3$ subtype-specific distribution was performed in the rat CNS using in situ hybridization. It was found that the three major isoforms are differentially expressed in the brain (Talley et al., 1999), likely accounting for the observed heterogeneity in pharmacology, modulation, and biophysics amongst native T-type currents (reviewed in (Huguenard, 1996)). For example, $\mathrm{Ca}_{\mathrm{v}} 3.1$ is predominantly expressed in thalamocortical relay cells (TCs) at high levels, while $\mathrm{Ca}_{\mathrm{v}} 3.2$ and $\mathrm{Ca}_{\mathrm{v}} 3.3$ are highly expressed in thalamic reticular (nRT) neurons. $\mathrm{Ca}_{\mathrm{v}} 3.1$ is also highly expressed in cerebellar PCs and inferior olive cells, while $\mathrm{Ca}_{\mathrm{v}} 3.2$ is highly expressed in the dentate gyrus
and dorsal root sensory ganglia. $\mathrm{Ca}_{\mathrm{v}} 3.3$ subunits have a more limited distribution but are highly expressed in the olfactory bulb and in the CA1/CA3 layers of the hippocampus (Talley et al., 1999). Functional studies have now confirmed many of these specific $\mathrm{Ca}_{\mathrm{v}} 3$ subtype distribution patterns. Recordings on TCs from Ca $\mathrm{Ca}_{\mathrm{v}} 3.1$ knock-out (KO) mice revealed a complete loss of T-type currents (Kim et al., 2001), while RNAi-mediated knock-down of $\mathrm{Ca}_{\mathrm{v}} 3.2$ expression in dorsal root ganglion (DRG) sensory neurons severely attenuated "Cav332-like" functional currents (Bourinet et al., 2005).

A further observation from the initial in situ characterization was that only a few regions of the brain, such as the olfactory bulb granule cell layer and the CA1/CA3 layers of the hippocampus, displayed significant expression of all three T-type isoforms (Talley et al., 1999). Contrastingly, a recent immunohistochemical study of $\mathrm{Ca}_{\mathrm{v}} 3$ protein expression in the rat brain questions this finding, with the conclusions that all three isoforms are expressed in many neurons throughout the brain and that heterogeneity is predominantly restricted to subcellular differences in T-type isoform distribution (McKay et al., 2006). It is important to note that the latter study reported differences in immunolabeling between slices processed in parallel from the same animal, only examined brain regions known to express high levels of functional T-type currents and could not distinguish between T-type channel protein levels (McKay et al., 2006). Furthermore, where discrepancies exist between the Talley et al. mRNA study and the McKay et al. protein study, the bulk of literature often supports the Talley et al. findings. For example, the McKay et al. study showed that $\mathrm{Ca}_{\mathrm{v}} 3.3$ is the main $\mathrm{Ca}_{\mathrm{v}} 3$ subtype expressed in cerebellar PCs, with prominent $\mathrm{Ca}_{\mathrm{v}} 3.3$ expression throughout the soma and dendritic arbour and $\mathrm{Ca}_{\mathrm{v}} 3.1$ expression in only the soma (McKay et al., 2006; Molineux et al., 2006), while the Talley et al. study showed that $\mathrm{Ca}_{\mathrm{v}} 3.1$ is selectively expressed in PCs (Talley et al., 1999). Three other studies have shown prominent $\mathrm{Ca}_{\mathrm{v}} 3.1$ expression in PCs at the protein and mRNA levels, with $\mathrm{Ca}_{\mathrm{v}} 3.1$ protein present at high levels in the soma and dendrites (Craig et al., 1999; Kase et al., 1999; Yunker et al., 2003), and the biophysical properties of T-type currents in PCs resemble $\mathrm{Ca}_{\mathrm{v}} 3.1$ more than $\mathrm{Ca}_{\mathrm{v}} 3.3$ (Isope and Murphy, 2005). One other immunohistochemical comparison has been performed using purported subtypespecific $\mathrm{Ca}_{\mathrm{v}} 3.1$ and $\mathrm{Ca}_{\mathrm{v}} 3.3$ antibodies (Yunker et al., 2003). However, the antibody generated against $\mathrm{Ca}_{\mathrm{v}} 3.3$ has since been reported to be cross-reactive with the endogenous neural cell adhesion protein, NCAM-180 (Chen et al., 2007), providing a warning of the potential pitfalls when generating antipeptide antibodies for the staining of low abundance membrane proteins within brain slices. Overall, when exploring T-type channel expression it is critical to examine a combination of mRNA, protein and biophysical evidence (with KO or antisense studies being essential) prior to forming conclusions on the nature of the $\mathrm{Ca}_{\mathrm{v}} 3$ subtypes that are functionally expressed.

While $\mathrm{Ca}_{\mathrm{v}} 3.3$ expression is restricted to the nervous system, both the $\mathrm{Ca}_{\mathrm{v}} 3.1$ and $\mathrm{Ca}_{\mathrm{v}} 3.2$ isoforms are expressed in excitable and non-excitable cells outside of the nervous system. In fact, compared to all other human tissue, $\mathrm{Ca}_{\mathrm{v}} 3.2 \mathrm{mRNA}$ is expressed most abundantly within the kidney (Cribbs et al., 1998).

There is also expression of $\mathrm{Ca}_{\mathrm{v}} 3.2$ within the pituitary and pineal glands, liver, adrenal cortex, heart and sperm (Talley et al., 1999) (reviewed in (Darszon et al., 2006; Perez-Reyes, 2003)). The Cav 3.1 subunit is expressed in the ovaries, placenta and heart (reviewed in (Perez-Reyes, 2003)).

### 1.2.4 T-type channel physiological roles

Differences in biophysical properties, alternative splicing, and expression of individual $\mathrm{Ca}_{\mathrm{v}} 3$ subtypes all allow for considerable functional specialization within the nervous system. The overlap between the voltage-dependence of T-type activation and inactivation at potentials near neuronal resting membrane potentials (McRory et al., 2001) creates the possibility for "window currents", where a fraction of channels are tonically open at rest. In hippocampal CA1 neurons, voltage-gated $\mathrm{Ca}^{2+}$ channel activity has been shown to affect resting cytosolic $\mathrm{Ca}^{2+}$ concentrations, at least partly through the activity of T-type channels (Magee et al., 1996). The presence and relevance of T-type window currents (and Ttype currents in general) is highly dependent on the resting membrane potential, as the channels will become completely inactivated at more depolarized potentials (Carter and Sabatini, 2004). T-type window currents have the possibility of affecting $\mathrm{Ca}^{2+}$-mediated signaling pathways as well as the electrical firing patterns of neurons (Chevalier et al., 2006; Williams et al., 1997).

The predominant expression of T-type currents in neuronal dendrites implicates their potential involvement in signal integration at synaptic inputs. In both pyramidal cortical and hippocampal CA1 neurons, subthreshold excitatory postsynaptic potentials (EPSPs) can cause the activation of T-type currents and a resultant localized increase in dendritic $\mathrm{Ca}^{2+}$ levels (Magee et al., 1995; Markram and Sakmann, 1994). This T-type activity could act to boost dendritic depolarizations and therefore increase excitability, or conversely, could activate $\mathrm{Ca}^{2+}$-activated $\mathrm{K}^{+}$currents to cause membrane hyperpolarizations (Wolfart and Roeper, 2002).

Dendritic T-type $\mathrm{Ca}^{2+}$ currents can also affect intracellular $\mathrm{Ca}^{2+}$ signaling pathways, potentially leading to synaptic plasticity. In addition to the above subthreshold, localized T-type $\mathrm{Ca}^{2+}$ transients, robust activation of T-type channels can produce an active low-threshold $\mathrm{Ca}^{2+}$ spike that spreads to adjacent dendrites (Egger et al., 2005). T-type $\mathrm{Ca}^{2+}$ spikes that are dendritically generated and localized (propagate poorly to the soma) contribute to postsynaptic depolarization, $\mathrm{Ca}^{2+}$ entry and long-term potentiation (LTP) when hippocampal CA1 synapses are stimulated at high frequency (Golding et al., 2002). T-type channels are also implicated in the induction of associative long term potentiation within young granule cells of the adult hippocampus during similar theta burst induction protocols (SchmidtHieber et al., 2004). Conversely, low-frequency stimulation of CA1 synapses induces long-term depression (LTD) that is abolished with T-type antagonists ( $25 \mu \mathrm{M} \mathrm{Ni}^{2+}, 10 \mu \mathrm{M}$ nimodipine) (Christie et al., 1997). A form of LTP that occurs at synapses in the spinal cord during abnormal pain sensitivity
(hyperalgesia) is also dependent on T-type activity (Ikeda et al., 2003). Thus, T-type channels are implicated in modulating synaptic strength through either localized or more global dendritic $\mathrm{Ca}^{2+}$ signals in both the CNS and PNS. However, the use of imperfect pharmacological tools, such as $\mathrm{Ni}^{2+}$, in many studies makes these postulations still quite preliminary. More thorough investigations involving highresolution two-photon $\mathrm{Ca}^{2+}$ imaging, $\mathrm{Ca}_{\mathrm{v}} 3 \mathrm{KO}$ mice, $\mathrm{Ca}_{\mathrm{v}} 3 \mathrm{RNAi}$-mediated knock-down, and/or more specific T-type antagonists are required to help elucidate the exact physiological roles of dendritic T-type currents in plasticity and excitability.

In addition to modulating plasticity, T -type $\mathrm{Ca}^{2+}$ spikes can also have profound effects on neuronal excitability. Low-threshold $\mathrm{Ca}^{2+}$ spikes were first identified from brain slices of the inferior olive, where removal of T-type inactivation with hyperpolarization initiated a spontaneous "reboundburst" spike (Llinas and Yarom, 1981). T-type channels have now been shown to underlie regenerative low-threshold spikes and burst firing in neurons throughout the CNS, including in the thalamus, inferior olive, cerebellum, hippocampus, cortex, and neocortex (reviewed in (Huguenard, 1996)). In some neurons, low threshold spikes and burst firing can alter neuronal oscillations, causing the neuron to switch from high frequency tonic firing to a phasic mode with regular intervals of high frequency bursts of spikes (Diana et al., 2007; Suzuki and Rogawski, 1989). Within the thalamus, T-type mediated changes in rhythmic oscillations underlie physiological sleep-wake gating and pathophysiological epileptic absence seizure activity (see below).

Recent work has begun to unveil the individual functional contributions of the $\mathrm{Ca}_{\mathrm{v}} 3$ isoforms to neuronal excitability. In one voltage-clamp study on HEK cells expressing individual recombinant Ttype isoforms, various neuronal AP firing waveforms were employed to demonstrate that the slower inactivation rates and faster deactivation kinetics of $\mathrm{Ca}_{\mathrm{v}} 3.3$ channels likely contribute to sustained rhythmic electrical activities (Chemin et al., 2002). Indeed, overexpressing $\mathrm{Ca}_{\mathrm{v}} 3.3$ channels in the NG108-15 neuronal cell line induced spontaneous oscillations and repetitive AP firing with a concomitant increase in intracellular $\mathrm{Ca}^{2+}$ concentration (Chevalier et al., 2006). Conversely, the faster activation and inactivation kinetics and slower deactivation kinetics of $\mathrm{Ca}_{\mathrm{v}} 3.2$ and especially $\mathrm{Ca}_{\mathrm{v}} 3.1$ allow these T-type isoforms to have a greater role in burst firing activity (Chemin et al., 2002). A recent study purports that rebound burst firing within deep cerebellar nuclei ( DCN ) neurons is restricted to a subpopulation that exclusively expresses $\mathrm{Ca}_{\mathrm{v}} 3.1$ channels (Molineux et al., 2006). Additionally, T-type currents in DRG neurons primarily consisting of $\mathrm{Ca}_{\mathrm{v}} 3.2$ channels are responsible for low threshold spikes that are crowned with a repetitive burst of APs (Bourinet et al., 2005; White et al., 1989).

Of all brain regions, the physiological roles of T-type channels have been most thoroughly studied in the thalamus. Thalamic TC and nRT nuclei form a rebound bursting circuit that creates spindle waves which are crucial during slow-wave sleep. Reciprocal interactions between T-type currents in TC and nRT cells are critical for the initiation and maintenance of this cycle. During periods
of inactivity (sleep initiation), T-type-dependent bursts of APs in nRTs cause hyperpolarization of TCs via GABAergic connections. The hyperpolarizing currents that flow through dendritic ionotropic $\mathrm{GABA}_{\mathrm{A}}$ receptors and $\mathrm{GABA}_{\mathrm{B}}$-activated channels de-inactivate T-type channels to initiate TC burst firing. Excitatory glutamatergic synapses from TCs onto the ionotropic glutamate receptors of $n R T s$ reexcite the nRT cells and start the cycle again, causing $6-15 \mathrm{~Hz}$ rhythmic oscillations (reviewed in (Destexhe and Sejnowski, 2003; McCormick and Contreras, 2001)).

Low-threshold burst firing varies between nRT and TC cells, with bursts in nRT cells displaying an accelerando-decelerando pattern of APs and a slower rising phase than TC bursts, resulting in broader bursts (Huguenard and Prince, 1992). Recent evidence suggests that these functional differences are due to differential expression of $\mathrm{Ca}_{\mathrm{v}} 3$ isoforms. T-type currents are predominantly located near the soma of TC cells (Coulter et al., 1989) and entirely consist of Cav 3.1 channels (Kim et al., 2001). Knocking out $\mathrm{Ca}_{\mathrm{v}} 3.1$ completely abolishes burst firing in TC cells (Kim et al., 2001). In contrast, T-type currents are largest in the dendrites of nRT cells (Destexhe et al., 1996) and electrophysiological evidence indicates that they consist of $\mathrm{Ca}_{\mathrm{v}} 3.3$ channels in the dendrites, with a lower density of $\mathrm{Ca}_{\mathrm{v}} 3.2$ channels in the soma (Joksovic et al., 2005). T-type currents in nRT cells of $\mathrm{Ca}_{\mathrm{v}} 3.2 \mathrm{KO}$ mice are reduced, but not abolished, indicating that both $\mathrm{Ca}_{\mathrm{v}} 3.2$ and $\mathrm{Ca}_{\mathrm{v}} 3.3$ isoforms are functionally expressed (Joksovic et al., 2006). The slow onset and broadness of bursts in nRT neurons is proposed to be due to the slower activation and inactivation kinetics of their $\mathrm{Ca}_{v} 3.3$-mediated T-type currents (Huguenard and Prince, 1992). Blocking T-type currents with $\mathrm{Ni}^{2+}$ or volatile anesthetics abolishes low threshold bursts in nRT cells (Joksovic et al., 2005). The essential role of thalamic T-type currents in generating rhythmic sleep oscillations is illustrated in the observation that conditional KO of $\mathrm{Ca}_{\mathrm{v}} 3.1$ in the thalamus, but not the cortex, disrupts AP firing in vitro and destabilizes sleep in vivo (Anderson et al., 2005).

The expression of $\mathrm{Ca}_{\mathrm{v}} 3.1$ and $\mathrm{Ca}_{\mathrm{v}} 3.2$ isoforms in circulatory, endocrine, and reproductive tissues suggests that T-type channels also play physiological roles outside of the nervous system. The $\mathrm{Ca}_{\mathrm{v}} 3.2$ isoform is robustly expressed in the atrial and ventricular myocytes of developing heart tissue, but is generally restricted to expression within conduction Purkinje fiber cells and pacemaker cells in the adult heart of humans and other higher-order mammals (Rosati et al., 2007) (reviewed in (Vassort et al., 2006)). Although the biophysical properties of T-type currents appear to be suited for generating pacemaker potentials in the atrioventricular and sinoatrial nodes of the heart, experiments involving Ttype antagonists and $\mathrm{Ca}_{\mathrm{v}} 3.2 \mathrm{KO}$ mice have demonstrated that T-type channels can modify heart rate firing frequency but are not the primary pacemaker currents in the heart (L-type Ca ${ }^{2+}$ channels are the primary pacemaker currents) (Chen et al., 2003a) (reviewed in (Vassort et al., 2006)). However, Cav 3.2 channels play an essential role in the function of non-excitable coronary smooth muscle. The constitutively constricted coronary arterioles and focal myocardial fibrosis observed in $\mathrm{Ca}_{\mathrm{v}} 3.2 \mathrm{KO}$ mice
demonstrate that $\mathrm{Ca}_{\mathrm{V}} 3.2$ currents are essential for coronary arteriole relaxation, possibly through coupling to inhibitory $\mathrm{Ca}^{2+}$-dependent $\mathrm{K}^{+}$channels $\left(\mathrm{B}_{\mathrm{K}}\right)$ (Chen et al., 2003a).

In addition to their putative roles in coronary smooth muscle, the slow increases in basal intracellular $\mathrm{Ca}^{2+}$ concentrations that are mediated by T-type window currents are also implicated in the function of non-excitable endocrine, kidney and sperm cells. Within the adrenal cortex, both the angiotensin II-induced secretion of aldosterone from adrenal glomerulosa cells and the adrenocorticotropin hormone-induced secretion of cortisol from zona fasciculata cells are thought to require $\mathrm{Ca}_{\mathrm{v}} 3.2$-mediated window $\mathrm{Ca}^{2+}$ currents (Yao et al., 2006) (reviewed in (Perez-Reyes, 2003)). Ttype window currents are also implicated in regulating the renal efferent arteriole smooth muscle tone and resultant microcirculation levels within the kidney (reviewed in (Hayashi et al., 2007)). Finally, $\mathrm{Ca}_{\mathrm{v}} 3.2$-mediated $\mathrm{Ca}^{2+}$ window currents play a major role in the sperm acrosome reaction of egg fertilization and may also function in sperm motility and capacitation (reviewed in (Darszon et al., 2006)).

### 1.2.5 T-type channels in human disease

Of the three T-type isoforms, only $\mathrm{Ca}_{\mathrm{v}} 3.2$ has been directly linked to human disease. Recent studies have shown that missense mutations in $\mathrm{Ca}_{\mathrm{v}} 3.2$ directly contribute to certain forms of epilepsy and autism spectrum disorders.

Epilepsy is a general disorder of the nervous system that is primarily characterized by hyperexcitability and hypersynchronization of thalamic and cortical neuronal circuits. Idiopathic generalized epilepsy (IGE) is a major form of epilepsy that has no clear etiology, is partly caused by complex non-Mendelian genetics, and includes disorders such as juvenile myoclonic epilepsy, juvenile absence epilepsy, and childhood absence epilepsy. Absence epilepsy is characterized by brief seizures that cause impairments of consciousness through 3 to 6 Hz spike-and-wave discharges (SWDs) mediated by oscillations within the thalamocortical circuit (reviewed in (McCormick and Contreras, 2001)). As previously discussed in section 1.2.4, T-type channels play critical roles in rhythmogenesis within these circuits.

Correlative evidence linking nRT T-type activity with epileptic activity can be found in a rat model of absence epilepsy (GAERS). In acutely dissociated nRT (but not TC cells) from GAERS rats, T-type currents have significantly higher current amplitude than currents from the nRT of wt rats. The increase in nRT T-type current is both age-dependent and absent at birth, as are the occurrence of seizures and SWD activity in this model of absence epilepsy (Tsakiridou et al., 1995). Furthermore, succinimide antiepileptic drugs reduce both T-type currents and associated burst generation in TC and nRT cells (Huguenard and Prince, 1994), providing further evidence for the link between T-type currents
within thalamic neurons and pathophysiological SWDs. More significantly concerning human epilepsy, missense mutations have been identified in the $\mathrm{Ca}_{\mathrm{v}} 3.2$ channel gene of human patients with childhood absence epilepsy (Chen et al., 2003b). Extensive electrophysiological analysis of these mutations as well as other subsequently identified $\mathrm{Ca}_{\mathrm{v}} 3.2$ IGE mutations have demonstrated that most of the mutations increase T-type channel activity by altering biophysical properties or by increasing $\mathrm{Ca}_{\mathrm{v}} 3.2$ surface expression (Heron et al., 2007; Khosravani et al., 2004; Khosravani et al., 2005; Peloquin et al., 2006; Vitko et al., 2007; Vitko et al., 2005). Interestingly, a majority of the mutations occur in the Cav 3.2 channel domain I-II linker (reviewed in (Adams and Snutch, 2007)), which is implicated in channel gating and expression (see section 1.2.2). As $\mathrm{Ca}_{\mathrm{v}} 3.2$ channels are expressed within the nRT (Talley et al., 1999), the gain-of-function mutations associated with IGE could cause nRT hyperexcitability and an increase in low-threshold bursting that contributes to SWD generation (reviewed in (Khosravani and Zamponi, 2006)). However, IGEs such as childhood absence epilepsy have a complex polygenic etiology and some Cav 3.2 mutations have no detectable effects on channel properties (Peloquin et al., 2006; Vitko et al., 2005). Extensive alternative splicing of Cav 3.2 may cause some mutations to only be functionally relevant in specific splice variants (Fig. 1.4; (Zhong et al., 2006)). The recent identification of single nucleotide polymorphisms in $\mathrm{Ca}_{\mathrm{v}} 3.2$ that are associated with an increased risk of childhood absence epilepsy indicates that mutations in $\mathrm{Ca}_{\mathrm{v}} 3.2$ can increase susceptibility to IGE in certain ethnicities (Liang et al., 2007).

Although mutations in $\mathrm{Ca}_{\mathrm{v}} 3.1$ have not been associated with human epilepsy to date, $\mathrm{Ca}_{\mathrm{v}} 3.1$ channel activity is linked to epileptic activity. In $\mathrm{Ca}_{\mathrm{v}} 3.1 \mathrm{KO}$ mice, both low-threshold $\mathrm{Ca}^{2+}$ spikes and burst firing in response to hyperpolarizations are abolished within TCs, and SWDs that can be induced by baclofen (a GABA $\mathrm{B}_{\mathrm{B}}$ agonist) in wt mice are absent in the $\mathrm{Ca}_{\mathrm{v}} 3.1 \mathrm{KO}$ mice (Kim et al., 2001). Epileptic Cav2.1 P/Q-type KO or missense mutant mice that have characteristic SWDs and behavioral arrests also have increased T-type currents within TCs (Song et al., 2004; Zhang et al., 2002). In one example, the SWDs were abolished in $\mathrm{Ca}_{\mathrm{v}} 2.1 / \mathrm{Ca}_{\mathrm{v}} 3.1$ double KO animals (Song et al., 2004).

Compared to the association between $\mathrm{Ca}_{\mathrm{v}} 3.2$ mutations and IGE, the link between $\mathrm{Ca}_{\mathrm{v}} 3.2$ and autism is relatively unexplored. Neuroanatomical studies have revealed histological abnormalities in the limbic system and other CNS regions of autism patients, including the hippocampus, amygdala, cerebellum, and cortex; all regions of high T-type expression and function (reviewed in (Adams and Snutch, 2007)). A recent study by Splawski et al. identified missense mutations within the Cav 3.2 channel in 6 out of 461 Caucasian autism patients. These mutations occur in conserved S4, P loop, and C-terminus channel regions and cause alterations in channel biophysical and current density properties that are predicted to reduce overall neuronal firing (Splawski et al., 2006).

Although not related to specific channel mutations, T-type currents are also associated with the pathophysiology of pain signaling. Evidence from RNAi-mediated knock-down, $\mathrm{Ca}_{\mathrm{v}} 3.2 \mathrm{KO}$ mice and
pharmacological experiments reveal that T-type $\mathrm{Ca}_{\mathrm{v}} 3.2$ currents contribute to the excitability of peripheral nociceptors and spinal cord dorsal horn neurons, where they are implicated in pronociceptive mechanisms of somatic pain (reviewed in (Hildebrand and Snutch, 2006)) (Appendix 1). Blockade of peripheral T-type currents with multiple clinical agents and pharmacological antagonists has been shown to reduce various forms of acute and chronic pain (reviewed in (Snutch and David, 2006)). Conversely, experiments on $\mathrm{Ca}_{\mathrm{v}} 3.1 \mathrm{KO}$ mice indicate that this T-type channel isoform plays an antinociceptive role in visceral pain responses via its role in sensory gating within thalamic relay nuclei (Appendix 1).

### 1.3 Modulation of voltage-gated calcium channels

### 1.3.1 G-protein-coupled receptors

One mechanism of altering ion channel activity in the CNS is through the activation of GPCRs by various neurotransmitter and neuropeptide ligands. Unlike ionotropic receptors that directly conduct transmembrane currents, GPCRs act on effectors through G-protein-mediated intracellular signaling pathways. Despite enormous diversity in sequence and functions that range from olfaction to phototransduction to synaptic integration, all GPCRs have a well-conserved tertiary structure consisting of an extracellular N -terminus, seven transmembrane $\alpha$ helices connected by alternating intracellular and extracellular loops, and a cytoplasmic C-terminus. The selective coupling between specific ligands and the N -terminus, and specific G-proteins and cytoplasmic regions results in a wide array of specific receptor subtypes connected to a multitude of downstream cellular effectors. Two of the major subfamilies of GPCRs in the brain are the metabotropic glutamate receptors (mGluRs) and muscarinic acetylcholine receptors (mAChRs).

The mGluR subfamily was originally cloned in the early 1990's as a novel subfamily of GPCR that lacked amino acid sequence similarity with other conventional GPCRs, such as mAChRs. It took functional expression of the rat cerebellar cDNA in Xenopus oocytes followed by serial dilutions of the response-evoking cDNA mixtures to painstakingly clone the first member, mGluR1a (Houamed et al., 1991; Masu et al., 1991). The cloning of additional mGluR members revealed highly conserved regions among the mGluR subfamily that include the N -terminus, transmembrane domains, and intracellular and extracellular loops (reviewed in (Schoepp and Conn, 1993)). Eight members of the mGluR subfamily have been cloned to date and are classified into 3 groups based on sequence similarity; Group I includes mGluR1 and mGluR5, Group II includes mGluR2 and mGluR3 and Group III includes mGluR4 and mGluR5-8. Functional characterization within heterologous and native systems has revealed that Group I mGluRs couple to $\mathrm{G} \alpha_{q / 11}$ proteins and phospholipase C (PLC) activation, leading to the hydrolysis of phosphatidylinositol into diacylglycerol (DAG) and inositol-1,4,5-trisphosphate ( $\mathrm{IP}_{3}$ ) (reviewed in (Conn and Pin, 1997)). Both of these components function as intracellular signals, as DAG is responsible for
the activation of protein kinase $\mathrm{C}(\mathrm{PKC})$ and $\mathrm{IP}_{3}$ increases the intracellular $\mathrm{Ca}^{2+}$ concentration through activation of $\mathrm{Ca}^{2+}$ permeable receptors $\left(\mathrm{IP}_{3} \mathrm{Rs}\right)$ in the membrane of the endoplasmic reticulum. mGluR1 also couples to other G-proteins to increase cAMP formation and arachidonic acid release when expressed in non-neuronal cell-lines (Nakanishi, 1992). As observed between T-type isoforms, mGluR1 and mGluR5 have similar sequences and functional properties and are both expressed in various regions of the CNS, yet their expression patterns are largely complementary (Abe et al., 1992). Both Group II and Group III mGluRs reduce cytosolic cAMP levels by inhibiting adenylate cyclase and can decrease the intracellular $\mathrm{Ca}^{2+}$ concentration, but only Group III receptors also decrease cGMP levels (Hayashi et al., 1992) (reviewed in (Coutinho and Knopfel, 2002)).

Activation of mGluRs within the CNS causes a range of electrophysiological effects, including the alteration of both ligand-gated and voltage-gated channel activity. For example, mGluR activation can cause either the inhibition or activation of voltage-gated $\mathrm{K}^{+}$and $\mathrm{Ca}^{2+}$ channels, the activation of nonspecific cationic currents, the potentiation of glutamatergic ionotropic receptors and the opening of Gprotein activated $\mathrm{K}^{+}$channels (reviewed in (Anwyl, 1999)). Through these mechanisms, mGluR activity is implicated in modulating neuronal excitability, neurotransmitter release, synaptic integration and synaptic plasticity (reviewed in (Coutinho and Knopfel, 2002)). However, the precise intracellular pathways that link mGluR activation to specific effectors within native systems remain to be identified.

Similar to mGluRs, mAChRs are divided into groups based on their sequence similarity and downstream effector coupling. Of the five mAChRs cloned to date, M1, M3, and M5 are classified as Group I mAChRs that couple to $\mathrm{G} \alpha_{q / 11}$ to activate PLC and its downstream pathways. Besides the $\mathrm{IP}_{3}$ and DAG-mediated signals, the breakdown of membrane phosphatidylinositol-4,5-bisphosphonate (PIP ${ }_{2}$ ) by PLC can directly alter channel activity, as $\mathrm{PIP}_{2}$ can act to stabilize or inhibit voltage-gated channels (reviewed in (Suh and Hille, 2005)). Similar to mGluR1, Group I mAChRs have been shown to increase arachidonic acid levels by activating phospholipase $\mathrm{A}_{2}$ (reviewed in (Lanzafame et al., 2003)). The other two mAChRs, M2 and M4, are classified as Group II and couple to PTX-sensitive G $\alpha_{\mathrm{i} /}$ proteins that inhibit adenylate cyclase to decrease cAMP. Group II mAChRs can also activate G-protein-coupled $\mathrm{K}^{+}$ channels through a direct $\mathrm{G} \beta \gamma$-mediated pathway, leading to membrane hyperpolarization (reviewed in (Ishii and Kurachi, 2006)). mAChRs are implicated in similar types of ion channel modulation and resultant neuronal processes as mGluRs. Further, both mGluR and mAChR receptors can desensitize through uncoupling between the receptor and relevant G-proteins (timescale of $\sim 2$ to 3 minutes) or through phosphorylation-induced internalization of the receptor (timescale of $\sim 10$ to 30 minutes) (reviewed in (Ishii and Kurachi, 2006)).

Besides the variability in subtype and functional coupling of GPCRs, signaling heterogeneity is also generated in the nervous system through differential expression of specific G-protein isoforms.

There are four main families of the intracellular signaling $\mathrm{G} \alpha$ proteins $\left(\mathrm{G} \alpha_{\mathrm{i} / \rho}, \mathrm{G} \alpha_{\mathrm{s}}, \mathrm{G} \alpha_{\mathrm{q}}\right.$, and $\left.\mathrm{G} \alpha_{13}\right)$, yet 16 specific G $\alpha$ genes have been identified within these families (reviewed in (Tedford and Zamponi, 2006)). GPCR activation results in the release of cytoplasmic GTP-bound G $\alpha$ proteins from membranebound G $\beta \gamma$ complexes that can act directly on nearby effectors, such as ion channels (reviewed in (Dascal, 2001)). To date, there are five cloned subtypes of $\mathrm{G} \beta$ and 12 different $\mathrm{G} \gamma$ subunits (reviewed in (Tedford and Zamponi, 2006)).

### 1.3.2 Modulation of HVA calcium channels

The functional coupling between GPCR activation and $\mathrm{Ca}^{2+}$ channel activity was first studied over 25 years ago, when Kathleen Dunlap and Gerald Fischback demonstrated that various neurotransmitters decreased AP duration in cultured chick DRG neurons by inhibiting voltage-gated $\mathrm{Ca}^{2+}$ conductances (Dunlap and Fischbach, 1978, 1981). Subsequent characterization of various native GPCR-Ca ${ }^{2+}$ channel interactions revealed that GPCR activation typically inhibits neuronal $\mathrm{HVA} \mathrm{Ca}^{2+}$ channel activity and that this inhibition can occur through both membrane-delimited, voltage-dependent and cytoplasmic, voltage-independent pathways.

The $\mathrm{Ca}^{2+}$ currents responsible for neurotransmitter release within presynaptic terminals primarily consist of Cav2.1 P/Q-type channels in the CNS and Cav 2.2 N-type channels in the PNS. Studies that followed Dunlap and Fishbach's initial discovery demonstrated that both the P/Q-type and N-type currents could be inhibited by numerous neurotransmitters, including acetylcholine, dopamine, glutamate, norepinephrine and serotonin all acting through metabotropic receptors. Thus, it was shown that neurotransmitters themselves can inhibit further neurotransmitter release through the inhibition of presynaptic $\mathrm{Ca}^{2+}$ channels. This GPCR-mediated inhibition of $\mathrm{Ca}^{2+}$ channels is voltage-dependent, with stronger inhibition occurring at more hyperpolarized potentials. Either strong voltage-clamped depolarizations ( $>+100 \mathrm{mV}$ ) or rapid physiological trains of APs can temporarily reverse this voltagedependent inhibition to produce "facilitation" (reviewed in (Elmslie, 2003)). Single channel recordings in small, detached membrane patches demonstrated that voltage-dependent $\mathrm{Ca}^{2+}$ channel inhibition is membrane-delimited and independent of soluble cytoplasmic signals. The inhibition also involves a reduction in the open probability at the single channel level that produces the characteristic slowing of activation kinetics observed at the whole-cell level (Lipscombe et al., 1989). Furthermore, the voltagedependent inhibition of native $\mathrm{Ca}_{\mathrm{v}} 2$ currents is accompanied by a concomitant positive shift in the voltage-dependence of channel activation and is usually PTX-sensitive, indicating an involvement of $G \alpha_{i / \rho}$ proteins. However, the demonstration of voltage-dependent inhibition through PTX-insensitive pathways revealed that the type of $G \alpha$ subunit is not the critical intracellular signal (reviewed in (Elmslie, 2003)).

Terry Snutch and colleagues were the first to propose that voltage-dependent inhibition is due to the direct binding of $\mathrm{G} \beta \gamma$ subunits to $\mathrm{Ca}_{\mathrm{v}} 2$ channels independent of $\mathrm{G} \alpha$ activity (Bourinet et al., 1996a). Shortly thereafter, two groups demonstrated that injection or overexpression of G $\beta \gamma$ subunits can reconstitute voltage-dependent inhibition of P/Q-type and N-type channels in both neurons and heterologous systems (Herlitze et al., 1996; Ikeda, 1996). Subsequent studies identified both the Nterminus and domain I-II linker of $\mathrm{Ca}_{\mathrm{v}} 2$ channels as being essential for direct $\mathrm{G} \beta \gamma$ binding and voltagedependent inhibition (reviewed in (Dolphin, 2003; Tedford and Zamponi, 2006)) (Fig. 1.5). The binding of $\mathrm{G} \beta \gamma$ to the domain I-II linker was shown to be antagonized by the binding of the accessory $\mathrm{Ca}^{2+}$ channel $\beta$ subunit (Bourinet et al., 1996a) as well as by the phosphorylation of specific residues within the domain I-II linker by PKC (Hamid et al., 1999; Zamponi et al., 1997). Further characterization at the single channel level demonstrated that $\mathrm{G} \beta \gamma$ binding stabilizes the closed state of $\mathrm{Ca}_{\mathrm{v}} 2$ channels to create a "reluctant" state with a prolonged latency to first opening. The extent of voltage-dependent $\mathrm{Ca}^{2+}$ channel inhibition is highly variable within neuronal systems, likely due to differences in primary sequence, cellular electrical properties, and colocalization between specific GPCRs, G $\beta \gamma$ and $\mathrm{Ca}_{\mathrm{v}} 2$ channel subtypes (reviewed in (Dolphin, 2003; Tedford and Zamponi, 2006)).


Figure 1.5-Modulation sites of the $\alpha_{1}$ calcium channel subunit.
Labeled $\mathrm{Ca}^{2+}$ channel modulation sites (not comprehensive) include a binding site for the $\mathrm{Ca}^{2+}$ channel $\beta$ accessory subunit in the domain I-II linker of all HVA $\mathrm{Ca}^{2+}$ channels (region indicated by green line). $\mathrm{G} \beta \gamma$ binding to the N -terminus, domain I-II linker, and possibly the C -terminus of $\mathrm{Ca}_{\mathrm{v}} 2$ channels (region indicated by red line) causes voltage-dependent inhibition, which is antagonized by PKC binding to overlapping regions (not shown). $\mathrm{Ca}_{\mathrm{v}} 3.2$ T-type channels are inhibited by $\mathrm{G}_{2} \gamma$ proteins binding to the domain II-III linker, while binding of CAMKII to the domain II-III linker causes the potentiation of $\mathrm{Ca}_{\mathrm{v}} 3.2$ channels (region indicated by dark blue line). High affinity binding of $\mathrm{Ni}^{2+}$ or $\mathrm{Zn}^{2+}$ to $\mathrm{His}_{191}$ of
$\mathrm{Ca}_{\mathrm{v}} 3.2$ channels mediates a specific inhibition, while endogenous reducing agents (L-cysteine) selectively potentiate $\mathrm{Ca}_{\mathrm{v}} 3.2$ channels through relief of tonic $\mathrm{Zn}^{2+}$ blockade (light blue line).

Unlike membrane-associated voltage-dependent inhibition, voltage-independent inhibition of HVA $\mathrm{Ca}^{2+}$ channels can occur through multiple intracellular pathways and is functionally defined as a GPCR-mediated inhibition that does not exhibit facilitation or a characteristic slowing of channel activation kinetics. Voltage-independent inhibition is often mediated by PTX-insensitive G $\alpha_{q / 11}$ proteins and can occur through both fast (seconds) and slow (minutes) signaling pathways (Beech et al., 1992). This type of inhibition has been well characterized in peripheral neurons as it relates to the voltageindependent inhibition of N -type $\mathrm{Ca}^{2+}$ currents by mAChRs (see below). Besides mAChR-mediated effects, activation of ORL1 receptors within nociceptive DRG neurons causes the slow ( 30 minutes) internalization of N-type channels (along with the receptor) into cytoplasmic vesicles through a PKCdependent pathway (Altier et al., 2006). Alternatively, voltage-independent channel internalization can
occur within seconds, as observed for the $\mathrm{GABA}_{\mathrm{B}}$-mediated internalization of N -type channels through a tyrosine kinase-dependent pathway in chick DRGs (Tombler et al., 2006). Voltage-independent inhibition effects can include a component that is dependent upon the nature of the N-type channel splice-variant. For example, the $\mathrm{Ca}_{\mathrm{v}} 2.2$ channel isoform most highly expressed in nociceptive DRGs includes exon 37a in its C-terminus. A specific residue within this region appears to be phosphorylated via GPCR-mediated tyrosine kinase activity to allow for voltage-independent inhibition and a mechanism whereby GABA, opiates and other neurotransmitters inhibit nociceptive $\mathrm{Ca}^{2+}$ currents independent of firing intensity (Raingo et al., 2007). Besides the activity of classical cytoplasmic kinases, it has also recently been shown that the breakdown of membrane PIP $_{2}$ by PLC can underlie voltage-independent inhibition. Wu and coworkers first demonstrated that $\mathrm{P} / \mathrm{Q}-$ type $\mathrm{Ca}^{2+}$ channels are stabilized in the plasma membrane by $\mathrm{PIP}_{2}$ (Wu et al., 2002). Following this key discovery, the activation of PLC by $\mathrm{G} \alpha_{11}$ and subsequent breakdown of $\mathrm{PIP}_{2}$ was shown to be directly involved in the voltage-independent muscarinic inhibition of both N-type and L-type channels (Gamper et al., 2004; Liu and Rittenhouse, 2003; Liu et al., 2006). The possible involvement of downstream phospholipase $\mathrm{A}_{2}$ activation and arachidonic acid production in this inhibition pathway is currently in debate (reviewed in (Gamper and Shapiro, 2007)).

Muscarinic inhibition of $\mathrm{HVA} \mathrm{Ca}{ }^{2+}$ channels generally occurs through $\mathrm{Ga}_{\mathrm{i}^{\prime} 0}$-coupled M2/M4 receptors for the voltage-dependent pathway and through $\mathrm{G}_{\mathrm{q} / 11}$-coupled $\mathrm{M} 1 / \mathrm{M} 3 / \mathrm{M} 5$ receptors for voltage-independent pathways. In both PNS superior cervical ganglion (SCG) neurons and CNS striatal neurons, voltage-independent inhibition occurs through M1 receptors, PTX-insensitive G $\alpha$ proteins, and a $\mathrm{Ca}^{2+}$-sensitive intracellular pathway, while voltage-dependent inhibition occurs through M4 receptors and PTX-sensitive G $\alpha$ proteins (Beech et al., 1991; Bernheim et al., 1992; Howe and Surmeier, 1995). However, promiscuity also exists between mAChRs and the inhibition pathways. In rat hippocampal neurons, voltage-dependent inhibition of non-N-type $\mathrm{HVA} \mathrm{Ca}^{2+}$ currents surprisingly occurs largely through M3 receptors coupled to PTX-sensitive G-proteins while voltage-independent inhibition of Ntype currents in the same cells occurs through M2 receptors (Toselli and Taglietti, 1995). Unlike the selective inhibition of $\mathrm{Ca}_{\mathrm{v}} 2$ channels through the voltage-dependent pathway, voltage-independent inhibition can also be observed for L-type channels. Expression of recombinant mAChRs in NIH 3 T3 cells revealed that L-type currents are inhibited by M2/M4 receptors through a cAMP- (but not PKA) mediated pathway, while M1/M3/M5 receptors inhibit L-type channels through a PKC-dependent pathway (Pemberton and Jones, 1997). Recombinant Cav 1.2 currents are also inhibited by M1, M3, or M5 activation through a slow voltage-independent pathway that requires $G \alpha_{q / 11}$ activity but is insensitive to antagonists of phospholipases, protein kinases, and protein phosphatases (Bannister et al., 2002).

Analysis of GPCR-Ca ${ }^{2+}$ channel interactions in recombinant expression systems can lead to insights that are often difficult to reach in many native systems where multiple receptors, channels, and signaling pathways co-exist (see above). These insights can often be relevant to native signal integration. For example, co-expression of $\mathrm{Ca}_{\mathrm{v}} 2.2$ channels and M1 receptors in HEK cells demonstrated a fast voltage-dependent inhibition through G $\beta \gamma$ proteins and a slower voltage-independent inhibition mediated by $\mathrm{G}_{q^{\prime} / 1}$ proteins (Melliti et al., 2001). This both confirmed and clarified the inhibition observed for native N-type currents by M1 receptor activation in SCG neurons (Kammermeier et al., 2000). Studying the effects of recombinant M1, M3 and M5 receptor activation on Cav 2.3 currents in HEK cells revealed that all three receptors stimulate this R-type current through activation of $\mathrm{G} \alpha_{\mathrm{q}}$, DAG, and $\mathrm{Ca}^{2+}$-independent PKCs, while only M3 and M5 receptors cause robust $\mathrm{G} \beta \gamma$-mediated, voltage-dependent inhibition of $\mathrm{Ca}_{\mathrm{v}} 2.3$ currents prior to the stimulation (Bannister et al., 2004; Melliti et al., 2000). These results led MacVicar and colleagues to identify a similar stimulation of native R-type currents by M1/M3 receptor activation in hippocampal CA1 neurons that also occurs through a $\mathrm{Ca}^{2+}$ independent PKC pathway and ultimately alters neuronal firing patterns (Tai et al., 2006).

The modulation of $\mathrm{Ca}^{2+}$ channels by mGluRs has been studied much less thoroughly than for mAChRs. Like mAChRs, $\mathrm{G} \mathrm{\alpha}_{\mathrm{i} 0}$-coupled Group II mGluRs inhibit $\mathrm{HVA} \mathrm{Ca}^{2+}$ channels through a membrane-delimited, voltage-dependent pathway (McCool et al., 1996), while activation of $\mathrm{G} \mathrm{\alpha}_{q / 11^{-}}$ coupled Group I mGluRs cause voltage-independent inhibition of $\mathrm{Ca}_{\mathrm{v}} 2$ currents (McCool et al., 1998). Inhibition of HVA $\mathrm{Ca}^{2+}$ channels by mGluRs has been observed in various regions of the CNS, including hippocampal CA3 pyramidal cells (Swartz and Bean, 1992), dentate gyrus granule cells (Schumacher et al., 2000) and neostriatal neurons (Colwell and Levine, 1999). Activation of heterologously-expressed mGluR1a receptors in SCG neurons also induces similar effects on N-type currents to those observed for native M1 receptor activation, including $\mathrm{G} \beta \gamma$-mediated, voltage-dependent inhibition and $\mathrm{G} \alpha_{\mathrm{q} / 11}$ mediated, voltage-independent inhibition (Kammermeier and Ikeda, 1999). Also, as observed for Group I mAChRs (Bannister et al., 2004), activation of mGluR1a stimulates Cav 2.3 R-type channels through a PKC-mediated pathway (Stea et al., 1995).

The stimulation of $\mathrm{Ca}_{\mathrm{v}} 2.3$ currents by GPCR-mediated activation of PKC illustrates another form of $\mathrm{Ca}^{2+}$ channel modulation. Although voltage-dependent and -independent inhibition of HVA $\mathrm{Ca}^{2+}$ currents are more predominant, $\mathrm{Ca}^{2+}$ channels can also be potentiated by various intracellular kinases and signaling pathways. For example, both $\mathrm{Ca}_{\mathrm{v}} 1.2$ and $\mathrm{Ca}_{\mathrm{v}} 2.1$ currents can be facilitated through the association of $\mathrm{Ca}^{2+} /$ calmodulin complexes (Lee et al., 1999a) (reviewed in (Lee and Catterall, 2005)). Furthermore, besides $\mathrm{Ca}_{\mathrm{v}} 2.3$, PKC can also stimulate $\mathrm{Ca}_{\mathrm{v}} 2.2$ currents but has no effect on $\mathrm{Ca}_{\mathrm{v}} 2.1$ or $\mathrm{Ca}_{\mathrm{v}} 1.2$ currents expressed in oocytes (Stea et al., 1995). This PKC-mediated stimulation can antagonize G $\beta \gamma$-mediated inhibition in signaling cross-talk (Zamponi et al., 1997). Other kinases, such as protein
kinase A (PKA), $\mathrm{Ca}^{2+} /$ calmodulin kinase II (CAMKII), mitogen-activated protein kinase (MAPK), tyrosine kinases, and lipid kinases can also stimulate $\mathrm{HVA} \mathrm{Ca}^{2+}$ currents, but the potentiation effects are much more localized and specific than the general inhibition pathways that have been discussed (reviewed in (Bannister et al., 2005)).

### 1.3.3 Modulation of T-type calcium channels

T-type $\mathrm{Ca}^{2+}$ channels were originally thought to be resistant to modulation by intracellular signaling pathways, partly because of the lack of cell dialysis-induced T-type current rundown during whole-cell recordings. Subsequent studies on T-type modulation in native systems revealed a number of discrepancies, with individual neurotransmitter types being reported to inhibit, stimulate, or have no effect on T-type currents depending upon the tissue and cell type being examined (reviewed in (Yunker, 2003)). After a lull following their initial cloning and characterization, the study of recombinant T-type channel modulation is currently emerging as an essential tool aimed at shedding further light on modulation within native systems. Of the three T-type isoforms, $\mathrm{Ca}_{\mathrm{v}} 3.2$ appears to be the most widely modulated, with the domain II-III linker acting as a signaling hotspot. Contrastingly, very little is presently known concerning the modulation of $\mathrm{Ca}_{\mathrm{v}} 3.1$ and $\mathrm{Ca}_{\mathrm{v}} 3.3$ channels. Because of the vast diversity of neurotransmitter-activated GPCRs and the functional heterogeneity within each GPCR family, the modulation of T-type channels by all possible GPCRs will not be discussed in detail here (see (Chemin et al., 2006; Yunker, 2003) for extensive reviews). This section will focus on the modulation of T-type currents by downstream cellular signaling pathways, including G-proteins, kinases, and phosphatases. The effects of both endogenous reducing agents and bioactive lipids on T-type currents will also be discussed. This section will end with a discussion on our current known status concerning the modulation of T-type channels by mAChR- and mGluR-mediated pathways.

The $\mathrm{G} \beta \gamma$-mediated inhibition of $\mathrm{Ca}^{2+}$ channels was originally thought to be restricted to $\mathrm{Ca}_{\mathrm{v}} 2$ channels because of a lack of observed voltage-dependent inhibition for the L-type and T-type $\mathrm{Ca}^{2+}$ channel classes. However, the inhibition of LVA Ca ${ }^{2+}$ currents by dopamine D1 receptors in adrenal glomerulosa cells was shown to involve $\mathrm{G} \beta \gamma$ subunits (Drolet et al., 1997). In a pioneering paper within the field of T-type channel modulation, Wolfe et al. co-expressed specific G $\beta \gamma$ proteins with $\mathrm{Ca}_{\mathrm{v}} 3$ isoforms to demonstrate that $\mathrm{Ca}_{v} 3.2$, but not $\mathrm{Ca}_{\mathrm{v}} 3.1$, is specifically inhibited by $\mathrm{G} \beta \gamma$ proteins that contain $\mathrm{G} \beta_{2}$ (Wolfe et al., 2003). Experiments using $\mathrm{Ca}_{\mathrm{v}} 3.1-\mathrm{Ca}_{\mathrm{v}} 3.2$ chimeric channels and fusion proteins demonstrated that $\mathrm{G} \beta_{2} \gamma$ proteins directly bind to the domain II-III linker of $\mathrm{Ca} \mathrm{v}^{2} .2$ channels and that this binding is both necessary and sufficient for inhibition (Fig. 1.5). Unlike the G $\beta \gamma$-mediated inhibition of $\mathrm{Ca}_{\mathrm{v}} 2$ channels, the inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.2$ is voltage-independent and not associated with a concomitant change in channel kinetics or voltage dependence (Wolfe et al., 2003). A follow-up study using purified
recombinant $\mathrm{G} \beta \gamma$ subunits revealed that the $\mathrm{G} \beta_{2} \gamma$-mediated inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.2$ channels involves a reduction in the open probability of the channels, with no effects on membrane expression or activechannel gating properties, and also identified specific residues within the $\mathrm{G} \beta_{2}$ subunit that confer this specific inhibition (DePuy et al., 2006). We have recently linked this specific G $\beta \gamma$-mediated inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.2$ to activation of a native GPCR by showing that activation of the endogenous urocortin receptor, CRFR1, causes the selective inhibition of both rat and human $\mathrm{Ca}_{\mathrm{v}} 3.2$ currents through the $\mathrm{G} \beta \gamma$ mediated pathway (Tao et al., 2008) (Appendix 2).

The study of the modulation of T-type $\mathrm{Ca}^{2+}$ channels by CAMKII is a clear example of how experiments using recombinant subtypes can further our understanding of modulation within native systems. In the early 1990s, peak T-type currents levels were shown to be sensitive to intracellular $\mathrm{Ca}^{2+}$ concentration in cardiac cells (Tseng and Boyden, 1991). Shortly thereafter, Barrett and colleagues demonstrated that in bovine adrenal glomerulosa cells, T-type currents are potentiated by $\mathrm{Ca}^{2+}$-dependent CAMKII activity through a hyperpolarizing shift in their voltage-dependence of activation (Lu et al., 1994). Cell-attached patch recordings confirmed that CAMKII activation did indeed underlie the $\mathrm{Ca}^{2+}$ mediated augmentation of T-type currents by increasing the single channel open probability (Barrett et al., 2000). Upon the molecular cloning of the three $\mathrm{Ca}_{\mathrm{v}} 3$ subtypes, the potentiation of T-type currents by CAMKII activation was recapitulated in HEK cells by co-expression of $\mathrm{Ca}_{\mathrm{v}} 3.2$, but not $\mathrm{Ca}_{\mathrm{v}} 3.1$, with CAMKII $\gamma_{\mathrm{c}}$ (Wolfe et al., 2002). Similar to their study on $\mathrm{G} \beta \gamma$-mediated modulation, the Barrett lab used chimeric channels, point mutations, and fusion proteins to reveal that CAMKII specifically modulates $\mathrm{Ca}_{\mathrm{v}} 3.2$ channels by directly interacting with the domain II-III linker and phosphorylating a specific serine residue (Ser ${ }^{1198}$, Fig. 1.5) (Welsby et al., 2003). They then used the results from the HEK system to design immunohistochemical experiments in adrenal glomerulosa cells that demonstrated that activation of the Ang II receptor in vivo results in an increased phosphorylation of the Ser ${ }^{1198}$ residues of native $\mathrm{Ca}_{\mathrm{v}} 3.2$ channels (Yao et al., 2006). Thus, the potentiation of $\mathrm{Ca}_{\mathrm{v}} 3.2$ channels by CAMKII is functionally implicated in the AngII receptor-mediated regulation of aldosterone synthesis and secretion within adrenal cells.

In contrast to the specific effects of CAMKII on Cavv 3.2 channels, the effects of PKA and PKC on T-type currents are much less clear. Activation of GPCRs specific for growth hormones (Chen et al., 2000), serotonin (Kim et al., 2006), and acetylcholine (Pemberton and Jones, 1997) all cause an increase in T-type currents mediated by cAMP and downstream PKA activity. However, many of these modulation studies are either incomplete or have significant experimental limitations, such as the inappropriate use of small whole-cell currents ( $\sim 20 \mathrm{pA}$; low signal-noise ratio) for pharmacological studies (Chen et al., 2000). Recombinant $\mathrm{Ca}_{\mathrm{v}} 3.2$ channels are stimulated by PKA activity when expressed in Xenopus oocytes, but this effect has an extremely slow time course ( $>1$ hour) and could not be repeated in experiments using HEK cells (Kim et al., 2006). Over an even longer time course,
incubation of cultured rat chromaffin cells with cAMP in the media for three to five days induces high density expression of $\mathrm{Ni}^{2+}$-sensitive T-type currents that requires protein synthesis but is independent of PKA activity (Novara et al., 2004).

Activation of PKC has been reported to stimulate, inhibit or have no effect on native T-type currents, depending on the system examined (reviewed in (Chemin et al., 2006)). In rat DRG neurons and bovine adrenal glomerulosa cells, PKC activation causes an inhibition of native T-type currents (Rossier et al., 1995; Schroeder et al., 1990). Interestingly, the phorbol 12-myristate 13-acetate (PMA)induced inhibition observed in DRG neurons is only observed at temperatures close to the mammalian physiological range (Schroeder et al., 1990). Within recordings on Xenopus oocytes at room temperature, activation of PKC by PMA causes a slow potentiation of all $3 \mathrm{Ca}_{\mathrm{v}} 3$ isoforms with no effect on other properties including no changes in membrane expression (Park et al., 2006). A recent paper explored the variable PKA- and PKC-dependent effects on $\mathrm{Ca}_{\mathrm{v}} 3$ isoforms in oocytes and HEK cells and determined that potentiation by chemical-induction of these kinases occurs at physiological relevant temperatures $\left(37^{\circ} \mathrm{C}\right)$ within HEK cells, but not at room temperature, because of a proposed deficit in kinase trafficking to the plasma membrane at lower temperatures (Chemin et al., 2007a). However, the chemical activation of kinases by compounds such as PMA may be more temperature sensitive than endogenous GPCR pathways, as PMA failed to induce potentiation of $\mathrm{Ca}_{\mathrm{v}} 2.3$ currents at room temperature (Chemin et al., 2007a), but activation of M1 receptors triggered robust PKC-mediated stimulation of $\mathrm{Ca}_{\mathrm{v}} 2.3$ currents at room temperature in the same expression system (Bannister et al., 2004).

The activation of intracellular phosphatases can also modulate T-type channel activity. T-type currents in mouse spermatogenic cells are facilitated in a voltage-dependent manner through a tyrosine phosphatase-dependent pathway (Arnoult et al., 1997). Spermatogenic T-type currents are highly $\mathrm{Ni}^{2+}$ sensitive, suggesting that they likely consist of $\mathrm{Ca}_{\mathrm{v}} 3.2$ channels (Arnoult et al., 1998), and are proposed to be activated to a higher conductance state following tyrosine phosphatase activity (Arnoult et al., 1997). Conversely, blocking tyrosine kinase activity with genistein ( $>10 \mu \mathrm{M}$ ) caused inhibition of Ttype currents in NG180-15 cells (Morikawa et al., 1998). However, it has recently been shown that genistein directly inhibits Cav 3.1 T-type channels independently of its action on tyrosine kinases in this concentration range $\left(\mathrm{IC}_{50}=25 \mu \mathrm{M}\right)$ (Kurejova and Lacinova, 2006).

Redox modulation provides another well characterized mechanism for altering T-type activity and associated neuronal excitability within native systems. Todorovic and colleagues have shown that both recombinant and native DRG T-type currents consisting of Cav 3.2 channels are selectively and potently stimulated by the endogenous reducing agent, L-cysteine (Todorovic et al., 2001b). This enhancement of T-type currents by L-cysteine at physiologically relevant concentrations is associated
with an increase in burst firing and DRG excitability in vitro (Nelson et al., 2005) and hyperalgesia in vivo (Todorovic et al., 2001b). L-cysteine also increases the overall excitability and burst firing of thalamic nRT neurons through the enhancement of native Cav 3 .2-mediated T-type currents (Joksovic et al., 2006). The selective potentiation of $\mathrm{Ca}_{\mathrm{v}} 3.2$ channels by reducing agents appears to be due to chelation of endogenous $\mathrm{Zn}^{2+}$ ions that selectively bind to an extracellular histidine residue on $\mathrm{Ca}_{\mathrm{v}} 3.2$ channels, but not $\mathrm{Ca}_{\mathrm{v}} 3.1$ or $\mathrm{Ca}_{\mathrm{v}} 3.3$ channels (Fig. 1.5) (Nelson et al., 2007b). Thus, the redox-mediated enhancement of DRG T-type channels is in fact a removal of specific inhibition of Cav 3.2 channels by endogenous $\mathrm{Zn}^{2+}$ ions (Traboulsie et al., 2007). Related to this phenomenon, the endogenous redox agent, ascorbate (vitamin C), has recently been shown to selectively and potently inhibit $\mathrm{Ca}_{\mathrm{v}} 3.2$ channels in HEK cells and in native DRG and nRT neurons, revealing a novel mechanism of action of vitamin C in the PNS and CNS (Nelson et al., 2007a).

T-type channels have recently been shown to be inhibited by several classes of natural lipids. The endogenous cannabinoid, anandamide, inhibits all three $\mathrm{Ca}_{\mathrm{v}} 3$ isoforms at submicromolar concentrations independently from the activation of its endogenous GPCRs, CB1 and CB2, and their downstream second messenger pathways. Inside-out patch recordings with anandamide applied to the intracellular surface revealed that the inhibition is due to direct action on T-type currents and includes an acceleration of inactivation kinetics and a hyperpolarizing shift in the voltage dependence of inactivation (Chemin et al., 2001b). Arachidonic acid is an $\omega-6$ polyunsaturated fatty acid that is a product of anandamide hydrolysis and is produced by many GPCR pathways to act as an intracellular signaling agent. Like anandamide, arachidonic acid has been shown to inhibit both $\mathrm{Ca}_{v} 3.1$ and $\mathrm{Ca}_{\mathrm{v}} 3.2$ channels through a membrane-delimited pathway that includes both a reduction in channel availability as well as a negative shift in the voltage-dependence of inactivation (Talavera et al., 2004; Zhang et al., 2000). In another example, the common dietary $\omega-3$ cis-polyunsaturated fatty acids, DHA and LNA, have also been shown to inhibit native T-type currents through a similar mechanism (Danthi et al., 2005). A recent paper has tied these findings on "bioactive lipids" together through a systematic study that identified the hydroxyl group and alkyl chain of anandamide and the degree of unsaturation, but not alkyl chain length, of fatty acids as key determinants for $\mathrm{Ca}_{\mathrm{v}} 3$ channel inhibition (Chemin et al., 2007b).

Relevant to the present proposal, the activation of mAChRs has been variously shown to inhibit, stimulate, or have no effect on native T-type currents. Transfection of individual recombinant mAChRs into NIH 3T3 fibroblast cells revealed that both M3 and M5 receptors increase T-type current amplitudes, while activation of M2 and M4 receptors has no effect and M1 receptors cause an increase in T-type currents albeit only after blocking PKC activity (Pemberton et al., 2000). Thus, heterogeneity in T-type modulation appears partially dependent on the subtype of mAChR co-expressed. Activation of mAChRs with a general muscarinic agonist (carbachol) augments $\mathrm{Ni}^{2+}$-sensitive T-type currents via increasing their open probability in hippocampal CA3 pyramidal neurons through a PTX-insensitive
pathway (Fisher and Johnston, 1990; Toselli and Lux, 1989). Muscarinic agonists also stimulate $\mathrm{Ni}^{2+}$ insensitive T-type currents with kinetics similar to $\mathrm{Ca}_{\mathrm{v}} 3.1$ channels in hippocampal interneurons (Fraser and MacVicar, 1991). However, interpretation of muscarinic effects in the hippocampus is complicated by an incomplete pharmacology that often does not eliminate contaminating R-type currents, which are potently potentiated by M1/M3 receptor activation (Tai et al., 2006). Recordings on Cav 3 3.2-like currents in hen granulosa cells revealed a robust carbachol-mediated inhibition of T-type currents (Wan et al., 1996). Similarly, T-type currents in rat DRGs (primarily consisting of $\mathrm{Ca}_{\mathrm{v}} 3.2$ ) are inhibited by acetylcholine (Formenti and Sansone, 1991). In contrast, highly $\mathrm{Ni}^{2+}$-sensitive T-type currents in magnocellular cholinergic basal forebrain neurons are not affected by muscarinic agonists (Allen and Brown, 1993). These discrepancies illustrate the need to study the interactions between specific mAChRs and $\mathrm{Ca}_{\mathrm{v}} 3$ subtypes in more well-defined systems.

The modulation of T-type channels by mGluRs is even less well characterized. Thus far, one voltage-clamp study has shown that T-type currents within retinal ganglion cells can be enhanced by activation of mGluR2 through a G-protein-dependent, but CTX and PTX-insensitive pathway (Robbins et al., 2003). However, the biophysical properties of the recorded currents in this paper do not appear to be completely characteristic of T-type channels and the possibility of the currents being mediated by Rtype $\mathrm{Ca}_{\mathrm{v}} 2.3 \mathrm{Ca}^{2+}$ channels has not been ruled out. In isolated hippocampal dendritic segments, activation of mGluRs had no significant effect on the recorded T-type currents (Kavalali et al., 1997). The dependence of N -methyl D -aspartate (NMDA) receptor-independent LTP on both $\mathrm{Ni}^{2+}$-sensitive $\mathrm{Ca}^{2+}$ currents and mGluR1 activity provides indirect evidence of a possible interaction between mGluR1 and T-type currents (Wang et al., 1997), but more extensive experiments are needed on this putative receptor-channel interaction.

### 1.4 Cerebellar Purkinje cells

### 1.4.1 Cerebellum overview

One area of the CNS wherein both mGluR1 receptors and T-type channels are functionally relevant is the cerebellum. Cerebellar PCs express a robust T-type current that is predominantly localized to the dendritic tree (Isope and Murphy, 2005). mGluR1 receptors are also highly expressed at synaptic sites within the PC dendritic tree, and serve critical functions in modulating synaptic plasticity, retrograde signaling, excitability and synaptic pruning within PCs (reviewed in (Knopfel and Grandes, 2002)). The roles of both of these proteins in PC physiology will be further discussed in the following sections.

The cerebellum is a highly folded brain region lying between the brain stem and cortex that is involved in balance; motor planning, initiation, and execution; motor learning; and some forms of
cognition (reviewed in (Barlow, 2002; Boyden et al., 2004; Ghez and Thach, 2000; Ito, 2001)). The highly-conserved cerebellum allows vertebrates to perform complicated movements and to calibrate these movements in time and space as the body and surrounding environment continually change. Despite such sophisticated functions, the cerebellar cortex has a highly repeated, uniform laminar structure that is oriented in parasagittal planes and composed of three layers of well-defined cell types (Fig. 1.6A). The cerebellar PCs are responsible for the sole output of the cerebellar cortex. In the mature cerebellar cortex, the PCs align in a single layer (the Purkinje cell layer) with an extensive apical dendritic arbourization (forming the molecular layer) in the parasagittal plane. This elaborate dendritic tree is the largest and most highly branched of all neurons in the CNS, yet is generally confined to a single plane perpendicular to the long axis of the cerebellar folia. The innermost layer beneath the Purkinje cell layer is called the granule cell layer and is composed of excitatory granule cells (GCs) and inhibitory golgi cells. Because of their small size and high density in the cerebellar cortex, GCs compose approximately half the neurons in the entire brain (reviewed in (Boyden et al., 2004)). The excitatory axons of GCs pass through the PC layer and bifurcate in the molecular layer, where they run perpendicular to the PC dendritic tree and parallel to the long axis of the cerebellar folia, giving them their name: parallel fibers (PFs). The PFs can pass through up to $\sim 1,000$ PC dendritic trees and the distal dendrites of individual PCs have a plethora of PF synaptic connections, ranging from approximately 175,000 in rats to 15 million PF synapses in humans (reviewed in (Altman and Bayer, 1997; Harvey and Napper, 1991)). The molecular layer also consists of inhibitory interneurons called stellate cells and basket cells that form GABAergic connections with the PC dendritic shafts or axon hillock, respectively.

The CNS and PNS are connected to the cerebellum through two well-defined excitatory inputs. The first is formed by moderately thin, myelinated axons called climbing fibers (CFs) that pass directly from the inferior olive of the medulla, through the white matter and granule cell layer, to the PC's proximal dendrites (Fig. 1.6B). Individual CFs branch and innervate 10 to 15 PCs (there are over 10 times less cells in the inferior olive compared to PCs), but a single PC is only innervated by a single CF at maturity, due to pruning of multiple CF innervations during the first few weeks of postnatal development. The CF wraps around the primary and secondary proximal dendrites of the PC in the inner two-thirds of the molecular layer and forms $\sim 1,500$ synaptic contact sites with a high release probability (reviewed in (Schmolesky et al., 2002)). The other main excitatory input carries both sensory and motor signals from various regions of the CNS and PNS to the cerebellum through brainstem relays in the pontine and reticular nuclei (Fig. 1.6B). The axons from these nuclei are called mossy fibers (MFs) and are thick and heavily myelinated. The MFs branch extensively and directly synapse with GC dendrites. The cluster of nerve endings at the end of the MFs form giant presynaptic terminal rosettes called glomeruli that each form excitatory glutamatergic synaptic connections with the dendrites of many GCs,
resulting in an individual MF being electrically connected to hundreds of GCs. The GCs then relay the MF signal to multiple PCs via their PFs (Fig. 1.6). The output of the cerebellum consists of PC axons projecting through the granule cell layer and white matter to make inhibitory GABAergic connections with several neurons of the DCN. Collaterals from the excitatory CF and MF cerebellar inputs also directly synapse with DCN neurons to generate output signals that are subsequently modulated by inhibitory PC activity (reviewed in (Ito, 2001)). Signals from the DCN neurons are relayed to the motor cortex via the thalamus, and are thought to be the start of the motor command pathway (reviewed in (Barlow, 2002)).

The unique repeating functional units within the cytoarchitecture of the cerebellar cortex create an ideal model system for studying physiological and molecular mechanisms of learning within the CNS. As each of these units contain the same cell types that are synaptically connected in the same manner (see above), it is presumed that the precise inputs and outputs of these units will vary depending on location within the cerebellum but the mechanisms of signal processing will remain constant. Therefore, studying a simple model of motor learning can reveal important insights into the underlying mechanisms of more complex motor learning behaviors (reviewed in (Boyden et al., 2004; Ito, 2001)). Many simple motor-learning paradigms, such as eyeball movement reflexes (e.g. - the vestibulo-ocular reflex), have now been well characterized at the underlying cerebellar cellular circuitry level. A distinct type of cellular plasticity called long-term depression (LTD) has also been thoroughly studied at the PF-PC synapse. As discussed in further sections, these cerebellar features allow for the linkage between molecular signaling mechanisms, synaptic plasticity and in vivo learning (reviewed in (Evans, 2007)).


Figure 1.6-Circuitry within the cerebellar cortex.
A) Three-dimensional configuration of the cerebellar cortex. The soma of PCs (brown) form the Purkinje cell layer, while the extensive dendritic arbourizations of the PCs are oriented in the parasagittal plane and form the molecular layer. Granule cells (GCs; light green) form the innermost granule cell layer. The axons of GCs (light green) project through the Purkinje cell layer to the molecular layer, where the bifurcate and run parallel to the long axis of the cerebellum. Inhibitory GABAergic interneurons, including stellate cells (blue), basket cells (red), and golgi cells (purple), are also found in
the cerebellar cortex and form synaptic connections with various cellular elements. B) The glutamatergic excitatory (green plus sign) inputs onto PCs (brown) consist of GC parallel fibers (PFs; light green) that synapse onto the distal dendrites of many PCs per individual PF and climbing fibers (CFs; dark green) that form thousands of synaptic connections with the proximal dendrites of the PC. Climbing fibers project directly from the inferior olive of the brainstem, while mossy fibers (MFs; yellow) from various brain regions project onto many GCs, which relay the excitatory signals to many

PCs via their PFs. The axon of PCs projects to deep cerebellar nuclei neurons (DCN; purple) via inhibitory (black minus sign) GABAergic synaptic connections and form the sole output of the cerebellum. DCN neurons relay signals to the motor cortex via the thalamus. Adapted with permission from Purves et al., 2001, Neuroscience, $2^{\text {nd }}$ edition, 416-417.

### 1.4.2 Cerebellar Purkinje cell physiology

The electrical and chemical activity of the cerebellar PC is shaped by strong but low frequency excitatory input from an individual CF and weak but high frequency excitatory inputs from multiple PFs. For simplicity, the effects of GABAergic interneurons at the PC axon hillock (basket cells) and dendritic tree (stellate cells) will not be discussed here (for more information, see (Evans, 2007)). Because the CF entwines the PC proximal dendrite and forms thousands of synaptic contacts, CF excitation results in the largest recorded EPSP ( $\sim 25 \mathrm{mV}$ ) yet observed in the CNS. Due to the amplitude of this EPSP, CF firing always results in a series of fast and slow postsynaptic spikes, termed the complex spike (reviewed in (Schmolesky et al., 2002)). This complex spike is evoked by glutamate binding to ligand-gated $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) ionotropic receptors to generate the large EPSP that causes sufficient depolarization to open voltage-gated $\mathrm{Ca}^{2+}$ channels and generate a putative dendritic $\mathrm{Ca}^{2+}$ spike (Knopfel et al., 1991). The putative $\mathrm{Ca}^{2+}$ spike in the PC dendrites excites voltagegated $\mathrm{Na}^{+}$channels that are predominantly localized in the soma, initiating the generation of a burst of somatic $\mathrm{Na}^{+}$-driven APs (Llinas and Sugimori, 1980a, b). The large $\mathrm{Ca}^{2+}$ transients that are generated by the CF-induced complex spike synchronously spread over the entire PC dendritic tree through the activation of dendritic $\mathrm{Ca}^{2+}$ channels (Miyakawa et al., 1992), proposed to consist of mainly P-type channels (Mintz et al., 1992; Usowicz et al., 1992; Watanabe et al., 1998). As the CF discharges at a low, irregular rate around 1 Hz that is much lower than the overall PC firing rate, the CF's contribution to the total spike output of PCs is small (reviewed in (Ito, 2002)). As we will discuss shortly, the CFinduced complex spike and dendritic $\mathrm{Ca}^{2+}$ transients serve additional purposes within the PC.

Although individual MFs excite many GCs which in turn excite (via their PFs) many PCs, the electrical excitation at an individual PF-PC synapse is quite small. Furthermore, a majority of the PF-PC synapses appear to be electrically silent (Isope and Barbour, 2002) in order to create well-defined PF receptive fields that are specifically coupled to the local CF input (Ekerot and Jorntell, 2001). The EPSPs that are produced at PF-PC synapses dissipate over the long distances of passive propagation between the distal dendrites, where they are generated, and the soma, where they are summated (Roth and Hausser, 2001). Temporal and/or spatial summation of distal PF-induced EPSPs is therefore required to induce PC firing (Brunel et al., 2004), and the conjunctive action of many MFs is needed to shape cerebellar PC output.

Mature PCs also exhibit spontaneous activity in the absence of PF and CF synaptic inputs. Much of this spontaneous activity is generated by the functional interplay between various voltage-gated ion channels in the PC dendrites (Womack and Khodakhah, 2002) (Swensen and Bean, 2003) that is independent of the back-propogation of somatic $\mathrm{Na}^{+}$-driven APs (Womack and Khodakhah, 2004). Like PC firing in vivo, this spontaneous in vitro firing can involve different modes and can reach rates of over 100 Hz . The spontaneous activity in PCs includes periods of rhythmic firing as well as periods of burst
firing, separated by silent periods (Womack and Khodakhah, 2002). This "trimodal" spontaneous firing pattern could be related to the membrane bistability that is observed under certain in vivo conditions (Loewenstein et al., 2005), with CF inputs controlling the transitions between firing states (McKay et al., 2007; Shin et al., 2007). Thus, the spontaneous activity of PCs will combine with the synaptic inputs to determine the overall firing output of PCs.

The electrical output of cerebellar PCs is also shaped by chemically-mediated changes in synaptic connections. The most well studied of these mechanisms is the LTD that occurs postsynaptically at the PF-PC synapse, termed PF-LTD. PF-LTD is produced by the activation of a bundle of PFs in conjunction with CF stimulation, leading to a sustained decrease in PF-PC EPSP amplitude (Ekerot and Kano, 1985; Ito et al., 1982). The reduction in EPSP amplitude can also be observed when an individual PF and PC are simultaneously depolarized during paired recordings (Casado et al., 2002). Electrophysiological and $\mathrm{Ca}^{2+}$ imaging experiments have shown that CF activity causes a widespread dendritic depolarization and resultant $\mathrm{Ca}^{2+}$ influx through voltage-gated $\mathrm{Ca}^{2+}$ channels necessary for PF-LTD (reviewed in (Eilers et al., 1996; Ito, 2002)). Thus, one model of motor learning suggests that the CF input to the PC provides a "teacher" signal that weakens synaptic inputs when error signals occur (reviewed in (Ito, 2001; Schmolesky et al., 2002)). The activation of PF-PC synapses also produces local $\mathrm{Ca}^{2+}$ signals, and the concurrent activation of PFs and CFs causes a supralinear increase in cytoplasmic $\mathrm{Ca}^{2+}$ that reaches up to $10 \mu \mathrm{M}$, which is much greater than the sum of the two independent $\mathrm{Ca}^{2+}$ inputs (Wang et al., 2000). Thus, some mechanism of "coincidence detection" creates a supralinear $\mathrm{Ca}^{2+}$ increase at the PF-PC synapse. The large increase in synaptic intracellular $\mathrm{Ca}^{2+}$ drives a kinase activation/ phosphatase suppression system that ultimately results in the reduction of functional AMPA receptors (GluR2/GluR3 subunits) in the postsynaptic density via direct phosphorylation by PKC and subsequent internalization (Linden, 2001; Linden and Connor, 1991; Xia et al., 2000). As AMPA receptors exclusively mediate glutamate-driven fast excitatory transmission at the PF-PC synapses (reviewed in (Eilers et al., 1996)), their internalization leads to the sustained reduction in EPSP amplitude that underlies PF-LTD.

Relevant to the importance of intracellular $\mathrm{Ca}^{2+}$ in PF-PC synaptic plasticity, $\mathrm{Ca}^{2+}$ levels are tightly regulated within PCs (reviewed in (Inoue, 2003)). High concentrations of endogenous $\mathrm{Ca}^{2+}$ buffers, such as calbindin and parvalbumin, are expressed in PC dendrites (Schmidt et al., 2003), which enables a rapid return to baseline $\mathrm{Ca}^{2+}$ levels after large dendritic $\mathrm{Ca}^{2+}$ transients (Sugimori and Llinas, 1990). One group has proposed that saturation of the large concentration of mobile, high-affinity $\mathrm{Ca}^{2+}$ buffer forms the coincidence detector behind the supralinear $\mathrm{Ca}^{2+}$ increase (Maeda et al., 1999). However, $\mathrm{Ca}^{2+}$ channels are also strongly implicated in this coincidence detector mechanism (Miyakawa et al., 1992; Wang et al., 2000).

The mechanism that links PF activity to PF-LTD is currently being investigated. Just as depolarization of the PC dendrite can replace the CF stimulation requirement for PF-LTD induction, so can glutamate application replace the PF stimulation requirement (reviewed in (Ito, 2002)). Glutamate activates both AMPA receptors and mGluRs at the PF-PC synapse, and the activation of mGluR1 has been shown to be an essential component of PF-LTD.

### 1.4.3 mGluR1 expression and function

The mGluR1 receptor was originally cloned from the cerebellum (Houamed et al., 1991; Masu et al., 1991) and subsequent in situ hybridization and immunohistochemical studies have confirmed a high level of mGluR1 expression in cerebellar PCs compared to other regions of the brain (Baude et al., 1993; Lin et al., 1997). The expression of mGluR1 increases in PCs during the first few weeks of development and remains at a high expression level in adult rodents while the expression of the other Group I mGluR, mGluR5, is very low or absent in the adult rat cerebellum (Casabona et al., 1997; Catania et al., 1994; Shigemoto et al., 1992). Viewing anti-mGluR1a stained cerebellar slices with a combination of light and electron microscopy has revealed that mGluR1a is expressed in the PC soma and dendrites of P10 to P12 rats, while mGluR1a expression increases and becomes localized to PC synapses in the molecular layer during later development (Lopez-Bendito et al., 2001). In the adult rat, mGluR1a is preferentially localized at the periphery of the PF-PC postsynaptic density (Baude et al., 1993; Lopez-Bendito et al., 2001). There is also evidence for expression of the mGluR1b isoform in a certain population of PCs (Mateos et al., 2000).

The study of intracellular signaling at the PF-PC synapse has progressed with the development of high resolution $\mathrm{Ca}^{2+}$ imaging techniques. Confocal laser scanning microscopy revealed that subthreshold excitation of PFs can induce $\mathrm{Ca}^{2+}$ transients in a PC dendritic branchlet and its associated spines, allowing for non-electrical synaptic integration (Eilers et al., 1995). Two-photon laser scanning microscopy experiments in the same year revealed that the smallest unit of integration within the PC is the dendritic spine, with PF-mediated activation of voltage-gated $\mathrm{Ca}^{2+}$ channels being localized to specific spines (Denk et al., 1995). A breakthrough in the understanding of PF-LTD was made when it was discovered that repetitive stimulation of PF synapses causes a localized $\mathrm{Ca}^{2+}$ transient in PC dendritic microdomains that is dependent on the activation of mGluR1 and the downstream $\mathrm{IP}_{3}$-mediated $\mathrm{Ca}^{2+}$ release from internal stores (Finch and Augustine, 1998; Takechi et al., 1998). This $\mathrm{Ca}^{2+}$ transient is specialized for local integration, as it is much more restricted than electrical signaling and ranges from confinement in individual spines to spinodendritic compartments depending on the level of stimulation (Takechi et al., 1998). The mGluR1-mediated $\mathrm{Ca}^{2+}$ transients at PF-PC synapses were shown to be a critical component of PF-LTD (Finch and Augustine, 1998), with some studies showing an absolute requirement of $\mathrm{IP}_{3}$-mediated $\mathrm{Ca}^{2+}$ release in this process (Miyata et al., 2000), while other studies
showing that PF-LTD can be elicited in the absence of $\mathrm{IP}_{3}$ signaling (Narasimhan et al., 1998). One study showed that (along with conjunctive activation of CFs) activation of a sparse number of PFs caused a supralinear $\mathrm{Ca}^{2+}$ increase restricted to single spines and mediated by an mGluR1 and $\mathrm{IP}_{3^{-}}$ dependent pathway. Meanwhile, activation of a dense PF bundle induced widespread LTD throughout dendritic branchlets that is mediated by voltage-gated $\mathrm{Ca}^{2+}$ channel activation and not mGluR1 or $\mathrm{IP}_{3}$ activity (Wang et al., 2000). Thus, both $\mathrm{IP}_{3}$-mediated $\mathrm{Ca}^{2+}$ release and $\mathrm{Ca}^{2+}$ channels are implicated in the $\mathrm{Ca}^{2+}$ signals that underlie PF-LTD.

In addition to $\mathrm{IP}_{3}$-mediated signaling, mGluR1 also contributes to local synaptic integration at PF-PC synapses through its activation of a slow excitatory postsynaptic current (sEPSC) (reviewed in (Knopfel and Grandes, 2002)). High frequency stimulation of PFs produces an mGluR1-mediated sEPSC and associated elevation in intracellular $\mathrm{Ca}^{2+}$ that is independent of $\mathrm{IP}_{3}$ receptor activation (Tempia et al., 1998) and is abolished in mGluR1 KO mice (Conquet et al., 1994). Subsequent analysis directly demonstrated that the sEPSC is mediated by cation-permeable ion channels that allow influx of both $\mathrm{Na}^{+}$and $\mathrm{Ca}^{2+}$ into PC dendrites (Tempia et al., 2001). Interestingly, the activation of the sEPSC by mGluR1 occurs independently from activation of PLC or PKC, but does require the activation of Gproteins and tyrosine phosphatases (Canepari and Ogden, 2003; Canepari et al., 2001; Hirono et al., 1998). Blockade of TRPC1 (transient receptor potential channel) activity with non-functional mutants or specific antibodies in the recording pipette attenuates the sEPSC in PCs, indicating that they are mediated by the TRPC1 cation channel (Kim et al., 2003). However, discrepancies in the single channel conductance and pharmacology between the native recorded sEPSC and TRPC1 currents indicate that other unidentified cationic channels may contribute to the sEPSC (Canepari et al., 2004). The potential direct contribution of this mGluR1-mediated sEPSC to physiological PF-LTD remains to be tested.

Along with in vitro experiments, mGluR1 activity has also been directly linked to PF-LTD in vivo. Cerebellar LTD recorded in vivo was verified to require the concomitant stimulation of CF and PF inputs (Ito et al., 1982) and was blocked with an mGluR1 antagonist (Gao et al., 2003). A direct linkage between in vivo PF-LTD and cerebellar motor learning was demonstrated in experiments on transgenic mice that specifically express a PKC antagonist in their cerebellar PCs. Both PF-LTD and adaptation of the vestibulo-ocular reflex are disrupted in these PKC-deficient transgenic animals (De Zeeuw et al., 1998). Studies on mGluR1 KO mice have also yielded exciting links between PF-LTD and in vivo motor learning. The mGluR1 KO mice exhibit severe deficits in motor coordination and spatial learning as well as impaired PF-LTD, yet they have no gross anatomical or basic electrophysiological impairments (Aiba et al., 1994; Conquet et al., 1994). Furthermore, injection of mGluR1-specific antibodies into the cerebellum of wt rats blocks the glutamate-induced sEPSC and PF-LTD (Shigemoto et al., 1994) and human Hodgkin's disease patients with autoantibodies against mGluR1 develop cerebellar ataxia due to a loss of PCs, a disruption of PF-LTD, and a resultant impairment of motor learning (Coesmans et al.,

2003; Sillevis Smitt et al., 2000). The most convincing evidence for a role of mGluR1 in the above processes is that rescuing the mGluR1 KO mice with expression of a mGluR1a transgene driven by a PC-specific promoter restores normal cerebellar LTD, interlimb coordination, and associative conditioning motor learning, while not affecting the deficits in non-spatial hippocampus-dependent learning (Ichise et al., 2000; Kishimoto et al., 2002).

It has also been demonstrated that mGluR1 is activated in response to CF stimulation and generates the same sEPSC and $\mathrm{IP}_{3}$-mediated $\mathrm{Ca}^{2+}$ increase as that observed at PF-PC synapses (Dzubay and Otis, 2002). The mGluR1-mediated sEPSC at CF synapses is first detectable during the second week of postnatal development, a period of CF pruning from multiple innervations to singular innervation (reviewed in (Altman and Bayer, 1997)). Aberrant multiple CF innervation has been observed in adult mice that have had either $\mathrm{mGluR1}, \mathrm{G} \alpha_{\mathrm{q}}, \mathrm{PLC} \beta 4$ or $\mathrm{PKC} \gamma$ genetically knocked out, indicating that the mGluR1-mediated signaling pathway is involved in the physiological process of CF pruning (Kano et al., 1997) (reviewed in (Hashimoto and Kano, 2005)). Abnormalities in CF synapse elimination first appear during the third postnatal week in the mGluR1 KO mice, indicating a role of the mGluR1 pathway in the late phases of CF pruning. This role was verified in recovery experiments where reintroducing mGluR1a expression specifically within the PCs of mGluR1 KO mice restores proper CF monoinnervation (Ichise et al., 2000). Besides their putative role in CF pruning, mGluR1 receptors also participate in the newly identified LTD that occurs at CF-PC synapses (Weber et al., 2003).

### 1.4.4 T-type expression and function

Compared to the well studied roles of mGluR1 in PC physiology, relatively little is known concerning the functional contributions of T-type $\mathrm{Ca}^{2+}$ channels. As discussed in section 1.2.3, the consensus from in situ hybridization and immunohistochemical experiments is that T-type channels are robustly expressed in the soma and dendrites of PCs. The primary subtype appears to be $\mathrm{Ca}_{\mathrm{v}} 3.1$ with the potential expression of Cav 3.3 in a subset of neurons (Craig et al., 1999; Kase et al., 1999; McKay et al., 2006; Talley et al., 1999; Yunker et al., 2003). The biophysical properties of T-type currents recorded from PCs include low sensitivity to $\mathrm{Ni}^{2+}$, fast inactivation kinetics, and slow deactivation kinetics that are more characteristic of $\mathrm{Ca}_{\mathrm{v}} 3.1$ currents than either $\mathrm{Ca}_{\mathrm{v}} 3.2$ or $\mathrm{Ca}_{\mathrm{v}} 3.3$ currents (Isope and Murphy, 2005; Kaneda et al., 1990).

T-type $\mathrm{Ca}^{2+}$ channel activity was first identified in PCs using sharp electrode intracellular recordings in adult rat cerebellar slices. These recordings on mature PCs revealed a prominent inwardrectifying hyperpolarization-activated inward current (Ih) as well as a putative low threshold $\mathrm{Ca}^{2+}$ conductance that is de-inactivated by hyperpolarization (Crepel and Penit-Soria, 1986). The presence of T-type currents in mammalian PCs was initially controversial, as one single channel recording study
failed to identify LVA Ca ${ }^{2+}$ currents in acute cerebellar slices from adult guinea pigs (Usowicz et al., 1992). T-type currents were however subsequently identified in PCs of both juvenile and adult rats and mice through recordings on acutely dissociated and primary cultured PCs, PCs from slice cultures and PCs from acute brain slices (Bossu et al., 1989; Gruol et al., 1992; Hirano and Hagiwara, 1989; Isope and Murphy, 2005; Kaneda et al., 1990; Mouginot et al., 1997; Raman and Bean, 1999; Watanabe et al., 1998). These native PC currents possess all of the hallmark T-type $\mathrm{Ca}^{2+}$ channel biophysical properties, including low threshold activation ranging between -60 mV and -40 mV , small single channel conductance between 7 and 9 pS , relatively hyperpolarizing voltage dependence of inactivation, fast activation and inactivation kinetics, and relatively slow deactivation kinetics (Bossu et al., 1989; Hirano and Hagiwara, 1989; Isope and Murphy, 2005; Kaneda et al., 1990; Mouginot et al., 1997). Cellattached recordings on PCs from newborn rat slice cultures demonstrated that T-type currents are distributed more densely on the dendritic membranes compared to the somatic membrane (Mouginot et al., 1997). This finding is consistent with the observation that T-type currents are only present in PCs that have developed a dendritic structure in a cell culture model of PC development (Gruol et al., 1992). A recent study by Isope and Murphy furthered these investigations by using a combination of two photon $\mathrm{Ca}^{2+}$ imaging and voltage-clamp recordings on PCs from juvenile rat acute brain slices to show that Ttype $\mathrm{Ca}^{2+}$ currents are present in both the spines and dendrites of PCs (Isope and Murphy, 2005). These T-type currents have a large peak amplitude (>-2 nA) that increases with developmental age, demonstrating that T-type currents are functionally expressed in adult rodent PCs (Isope and Murphy, 2005). Several studies have now reached the consensus that P-type and T-type currents comprise the vast majority (up to $95 \%$ ) of $\mathrm{Ca}^{2+}$ channel currents in cerebellar PCs (Isope and Murphy, 2005; Swensen and Bean, 2003; Usowicz et al., 1992; Watanabe et al., 1998).

Recent studies have implicated T-type $\mathrm{Ca}^{2+}$ channels as having a significant physiological role in dendritic $\mathrm{Ca}^{2+}$ spikes and burst firing within the soma and proximal dendrites of PCs (reviewed in (Cavelier and Bossu, 2003)). For over 25 years it has been generally accepted that $\mathrm{Na}^{+}$-driven APs are produced at the PC soma while $\mathrm{Ca}^{2+}$-driven APs are produced in the PC dendrites and that both are generally restricted to their respective compartments (Llinas and Sugimori, 1980a, b). In accordance with this notion, $\mathrm{Na}^{+}$-driven APs that are initiated in the axosomatic region of PCs (of a rat cerebellar slice culture) propagate very poorly into the dendritic tree, exponentially decreasing in amplitude with distance from the soma (Pouille et al., 2000). However, $\mathrm{Ca}^{2+}$-driven dendritic low threshold spikes only decrease in a linear manner as they approach the soma and can propagate directly to the soma in $\sim 20 \%$ of PCs examined (Pouille et al., 2000). The dendritic $\mathrm{Ca}^{2+}$ spikes originally studied in guinea pig PCs include both $\mathrm{Ca}^{2+}$-dependent plateau potentials as well as $\mathrm{Ca}^{2+}$ spikes (Llinas and Sugimori, 1980a, b), and P-type channels are proposed to generate these $\mathrm{Ca}^{2+}$ spikes (Usowicz et al., 1992; Watanabe et al., 1998). Examining PCs from rat organotypic cerebellar slice cultures revealed that T-type $\mathrm{Ca}^{2+}$ channels
underlie dendritic $\mathrm{Ca}^{2+}$ spikes while P -type $\mathrm{Ca}^{2+}$ channels underlie a plateau potential that is unmasked when $\mathrm{K}^{+}$channels are blocked (Pouille et al., 2000). In fact, pharmacological blockade of the P-type current promoted propagation of the low threshold $\mathrm{Ca}^{2+}$ spikes to the PC soma (increased from $20 \%$ to $80 \%$ of PCs) (Cavelier et al., 2002b). The robust P-type dendritic currents in PCs have been shown to activate $\mathrm{Ca}^{2+}$-dependent BK and $\mathrm{SK} \mathrm{K}{ }^{+}$channels, which induces after-hyperpolarizing potentials and alters the frequency of PC firing (Edgerton and Reinhart, 2003; Womack et al., 2004). It is proposed that dendrosomatic propagation of T-type-dependent $\mathrm{Ca}^{2+}$ spikes is inhibited by this activation of $\mathrm{Ca}^{2+}$ dependent $\mathrm{K}^{+}$channels, and that the low threshold spikes may underlie the CF-induced complex spike (Cavelier et al., 2003; Cavelier et al., 2002b). In separate experiments on acutely dissociated PCs and PCs from acute cerebellar slices, it has been shown that P-type currents are required to sustain the spontaneous firing of PCs, while T-type currents make a substantial contribution to $\mathrm{Ca}^{2+}$ currents generated during interspike intervals of spontaneous bursting (Swensen and Bean, 2003; Womack and Khodakhah, 2004).

Although the above studies implicate T-type $\mathrm{Ca}^{2+}$ channels in generating $\mathrm{Ca}^{2+}$-dependent bursting in PC dendrites, with potentially significant physiological implications, several limitations of these studies should be noted. Firstly, the overall structure and native composition of PCs is not well maintained in most of the in vitro systems currently used. Acutely dissociated PCs lack the dendritic tree where T-type $\mathrm{Ca}^{2+}$ channels predominate (Swensen and Bean, 2003), while cerebellar slice cultures are taken from newborn rats and subsequent dendritic growth and channel expression is determined by in vitro culture conditions (Cavelier et al., 2002a; Cavelier et al., 2003; Cavelier et al., 2002b; Pouille et al., 2000). Furthermore, most electrophysiological recordings have been performed in current clamp mode, where a lack of high affinity, specific T-type antagonists limits the conclusions that can be made. A thorough investigation into the role of T-type $\mathrm{Ca}^{2+}$ channels in generating dendritic burst firing and altering excitability within intact PCs of acute brain slices has been lacking, likely due to the spaceclamp complications that arise in adult PCs.

To date, no published study has examined the functional roles of T-type $\mathrm{Ca}^{2+}$ channels in distal dendritic PF-PC synapses. The supralinear $\mathrm{Ca}^{2+}$ increase required for PF-LTD implicates the possible involvement of $\mathrm{Ca}^{2+}$ channels due to their highly nonlinear dependence of activation on membrane potential. It has been shown that activation of AMPA receptors at PF synapses can cause sufficient depolarization to open voltage-gated $\mathrm{Ca}^{2+}$ channels (Denk et al., 1995; Ito, 2002), and coincident depolarization due to the CF-mediated complex spike would likely increase open probability. It is also known that mGluR1 can directly bind to P/Q-type $\mathrm{Ca}^{2+}$ channels and TRPC1 channels and is colocalized with both of these in PC dendrites (Kim et al., 2003; Kitano et al., 2003). However, the majority of research on dendritic signaling and plasticity in PCs has involved synaptic stimulation, current clamp recordings, and $\mathrm{Ca}^{2+}$ imaging and the direct contributions of low voltage-activated $\mathrm{Ca}^{2+}$ channels in these
processes is poorly understood. The potential interaction within PC distal dendrites between mGluR1 receptors and nearby T-type $\mathrm{Ca}^{2+}$ channels has significant implications concerning cerebellar integration, excitability, and plasticity.

### 1.5 Thesis hypotheses and objectives

### 1.5.1 Hypotheses

T-type $\mathrm{Ca}^{2+}$ channel isoforms have very distinct expression patterns in the mammalian CNS at both the cellular and subcellular levels which are likely correlated to their colocalization with different neurotransmitter receptor subtypes and downstream intracellular signaling pathways. This selective combination of coupling between T-type $\mathrm{Ca}^{2+}$ channel isoforms and signaling pathways is hypothesized to form the basis for T-type $\mathrm{Ca}^{2+}$ channels serving unique physiological functions in the CNS. In this thesis I specifically hypothesized:

1) that the three major Cav 3 T-type isoforms are differentially modulated by the activation of specific GPCRs,
2) that functional T-type currents in cerebellar PCs mainly consist of $\mathrm{Ca}_{v} 3.1$ channels, and
3) that in PCs the $\mathrm{Ca}_{\mathrm{v}} 3.1$-mediated T-type currents are modulated by mGluR1 and that this pathway serves unique roles in neuronal excitability and synaptic integration.

### 1.5.2 Objectives

In order to address the above hypotheses, the following scientific objectives were generated:

1) To study the functional effects of muscarinic and glutamatergic $\mathrm{G} \alpha_{q / 11}$-protein coupled receptor activation on specific T-type isoforms in a heterologous system in order to identify possible modulatory interactions. Any observed modulation of recombinant T-type channels will be characterized in terms of its effects on the channels' biophysical properties, the regions of the T-type channel that are involved in the modulatory interaction, and the intracellular signaling pathway mediating the effect.
2) To perform whole-cell voltage-clamp recordings on PCs from acute cerebellar slices of rats and $\mathrm{Ca}^{2+}$ channel KO mice to determine what specific $\mathrm{Ca}_{\mathrm{v}} 3$ isoform underlies functional T-type currents within PCs. The biophysical, pharmacological, and modulatory profile of the PC T-type currents will also be analyzed to complement the KO experiments in forming a firm conclusion.
3) To apply the information gained from Objective 1 to a native neuronal system where a specific T-type isoform and GPCR are functionally expressed: cerebellar PCs. Specific pharmacological antagonists and agonists will be used in the same cerebellar recordings as in Objective 2 to investigate the modulation of T-type currents by mGluR1 and the second messenger pathway that mediates this modulation. The
biophysical characteristics of the T-type modulation will be investigated with various voltage-clamp protocols and two-photon $\mathrm{Ca}^{2+}$ imaging will be used to determine the localization of the modulation. Combining current clamp recordings and synaptic PF stimulation with the above techniques will enable the investigation into how the observed T-type modulation affects excitability and local signaling within PCs.

### 1.6 References

Abe, T., Sugihara, H., Nawa, H., Shigemoto, R., Mizuno, N., and Nakanishi, S. (1992). Molecular characterization of a novel metabotropic glutamate receptor mGluR5 coupled to inositol phosphate/Ca2+ signal transduction. J Biol Chem 267, 13361-13368.

Adams, P.J., and Snutch, T.P. (2007). Calcium channelopathies: voltage-gated calcium channels. In Calcium signalling and disease - molecular pathology of calcium, E. Carafoli, and M. Brini, eds. (New York: Springer), pp. 215-251.

Aiba, A., Kano, M., Chen, C., Stanton, M.E., Fox, G.D., Herrup, K., Zwingman, T.A., and Tonegawa, S. (1994). Deficient cerebellar long-term depression and impaired motor learning in mGluR1 mutant mice. Cell 79, 377-388.

Akaike, N., Kostyuk, P.G., and Osipchuk, Y.V. (1989). Dihydropyridine-sensitive low-threshold calcium channels in isolated rat hypothalamic neurones. J Physiol 412, 181-195.

Allen, T.G., and Brown, D.A. (1993). M2 muscarinic receptor-mediated inhibition of the $\mathrm{Ca}^{2+}$ current in rat magnocellular cholinergic basal forebrain neurones. J Physiol 466, 173-189.

Altier, C., Khosravani, H., Evans, R.M., Hameed, S., Peloquin, J.B., Vartian, B.A., Chen, L., Beedle, A.M., Ferguson, S.S., Mezghrani, A., et al. (2006). ORL1 receptor-mediated internalization of N-type calcium channels. Nat Neurosci 9, 31-40.

Altman, J., and Bayer, S.A. (1997). Development of the cerebellar system : in relation to its evolution, structure, and functions (Boca Raton: CRC Press).

Anderson, M.P., Mochizuki, T., Xie, J., Fischler, W., Manger, J.P., Talley, E.M., Scammell, T.E., and Tonegawa, S. (2005). Thalamic Cav 3.1 T-type $\mathrm{Ca}^{2+}$ channel plays a crucial role in stabilizing sleep. Proc Natl Acad Sci U S A 102, 1743-1748.

Anwyl, R. (1999). Metabotropic glutamate receptors: electrophysiological properties and role in plasticity. Brain Res Brain Res Rev 29, 83-120.

Arias, J.M., Murbartian, J., Vitko, I., Lee, J.H., and Perez-Reyes, E. (2005). Transfer of beta subunit regulation from high to low voltage-gated Ca2+ channels. FEBS Lett 579, 3907-3912.

Arias, O., II, Vitko, I., Fortuna, M., Baumgart, J.P., Sokolova, S., Shumilin, I.A., Van Deusen, A., Soriano-Garcia, M., Gomora, J.C., and Perez-Reyes, E. (2008). Characterization of the Gating Brake in the I-II Loop of Cav3.2 T-type Ca2+ Channels. J Biol Chem 283, 8136-8144.

Arikkath, J., and Campbell, K.P. (2003). Auxiliary subunits: essential components of the voltage-gated calcium channel complex. Curr Opin Neurobiol 13, 298-307.

Arnoult, C., Lemos, J.R., and Florman, H.M. (1997). Voltage-dependent modulation of T-type calcium channels by protein tyrosine phosphorylation. Embo J 16, 1593-1599.

Arnoult, C., Villaz, M., and Florman, H.M. (1998). Pharmacological properties of the T-type Ca2+ current of mouse spermatogenic cells. Mol Pharmacol 53, 1104-1111.

Bannister, R.A., Melliti, K., and Adams, B.A. (2002). Reconstituted slow muscarinic inhibition of neuronal ( $\mathrm{Ca}_{\mathrm{v}} 1.2 \mathrm{c}$ ) L-type $\mathrm{Ca}^{2+}$ channels. Biophys J 83, 3256-3267.

Bannister, R.A., Melliti, K., and Adams, B.A. (2004). Differential modulation of $\mathrm{Ca}_{\mathrm{v}} 2.3 \mathrm{Ca}^{2+}$ channels by Galpha ${ }_{q / 11}$-coupled muscarinic receptors. Mol Pharmacol 65, 381-388.

Bannister, R.A., Meza, U., and Adams, B.A. (2005). Phosphorylation-dependent regulation of voltagegated $\mathrm{Ca}^{2+}$ channels. In Voltage-gated calcium channels, G.W. Zamponi, ed. (New York: Landes Bioscience), pp. 168-182.

Barlow, J.S. (2002). The cerebellum and adaptive control (Cambridge, U.K. ; New York, NY: Cambridge University Press).

Barrett, P.Q., Lu, H.K., Colbran, R., Czernik, A., and Pancrazio, J.J. (2000). Stimulation of unitary Ttype $\mathrm{Ca}(2+)$ channel currents by calmodulin-dependent protein kinase II. Am J Physiol Cell Physiol 279, C1694-1703.

Baude, A., Nusser, Z., Roberts, J.D., Mulvihill, E., McIlhinney, R.A., and Somogyi, P. (1993). The metabotropic glutamate receptor (mGluR1 alpha) is concentrated at perisynaptic membrane of neuronal subpopulations as detected by immunogold reaction. Neuron 11, 771-787.

Beech, D.J., Bernheim, L., and Hille, B. (1992). Pertussis toxin and voltage dependence distinguish multiple pathways modulating calcium channels of rat sympathetic neurons. Neuron 8, 97-106.

Beech, D.J., Bernheim, L., Mathie, A., and Hille, B. (1991). Intracellular $\mathrm{Ca}^{2+}$ buffers disrupt muscarinic suppression of $\mathrm{Ca}^{2+}$ current and M current in rat sympathetic neurons. Proc Natl Acad Sci U S A 88, 652656.

Bernheim, L., Mathie, A., and Hille, B. (1992). Characterization of muscarinic receptor subtypes inhibiting $\mathrm{Ca}^{2+}$ current and M current in rat sympathetic neurons. Proc Natl Acad Sci U S A 89, 95449548.

Berrow, N.S., Brice, N.L., Tedder, I., Page, K.M., and Dolphin, A.C. (1997). Properties of cloned rat alpha1A calcium channels transiently expressed in the COS-7 cell line. Eur J Neurosci 9, 739-748.

Bossu, J.L., Fagni, L., and Feltz, A. (1989). Voltage-activated calcium channels in rat Purkinje cells maintained in culture. Pflugers Arch 414, 92-94.

Bourinet, E., Alloui, A., Monteil, A., Barrere, C., Couette, B., Poirot, O., Pages, A., McRory, J., Snutch, T.P., Eschalier, A., and Nargeot, J. (2005). Silencing of the Cav 3.2 T-type calcium channel gene in sensory neurons demonstrates its major role in nociception. Embo J 24, 315-324.

Bourinet, E., Soong, T.W., Stea, A., and Snutch, T.P. (1996a). Determinants of the G protein-dependent opioid modulation of neuronal calcium channels. Proc Natl Acad Sci U S A 93, 1486-1491.

Bourinet, E., Soong, T.W., Sutton, K., Slaymaker, S., Mathews, E., Monteil, A., Zamponi, G.W., Nargeot, J., and Snutch, T.P. (1999). Splicing of alpha 1A subunit gene generates phenotypic variants of P- and Q-type calcium channels. Nat Neurosci 2, 407-415.

Bourinet, E., Zamponi, G.W., Stea, A., Soong, T.W., Lewis, B.A., Jones, L.P., Yue, D.T., and Snutch, T.P. (1996b). The alpha 1E calcium channel exhibits permeation properties similar to low-voltageactivated calcium channels. J Neurosci 16, 4983-4993.

Boyden, E.S., Katoh, A., and Raymond, J.L. (2004). Cerebellum-dependent learning: the role of multiple plasticity mechanisms. Annu Rev Neurosci 27, 581-609.

Brunel, N., Hakim, V., Isope, P., Nadal, J.P., and Barbour, B. (2004). Optimal information storage and the distribution of synaptic weights: perceptron versus Purkinje cell. Neuron 43, 745-757.

Canepari, M., Auger, C., and Ogden, D. (2004). $\mathrm{Ca}^{2+}$ ion permeability and single-channel properties of the metabotropic slow EPSC of rat Purkinje neurons. J Neurosci 24, 3563-3573.

Canepari, M., and Ogden, D. (2003). Evidence for protein tyrosine phosphatase, tyrosine kinase, and Gprotein regulation of the parallel fiber metabotropic slow EPSC of rat cerebellar Purkinje neurons. J Neurosci 23, 4066-4071.

Canepari, M., Papageorgiou, G., Corrie, J.E., Watkins, C., and Ogden, D. (2001). The conductance underlying the parallel fibre slow EPSP in rat cerebellar Purkinje neurones studied with photolytic release of L-glutamate. J Physiol 533, 765-772.

Carbone, E., and Lux, H.D. (1984). A low voltage-activated, fully inactivating Ca channel in vertebrate sensory neurones. Nature 310, 501-502.

Carter, A.G., and Sabatini, B.L. (2004). State-dependent calcium signaling in dendritic spines of striatal medium spiny neurons. Neuron 44, 483-493.

Casabona, G., Knopfel, T., Kuhn, R., Gasparini, F., Baumann, P., Sortino, M.A., Copani, A., and Nicoletti, F. (1997). Expression and coupling to polyphosphoinositide hydrolysis of group I metabotropic glutamate receptors in early postnatal and adult rat brain. Eur J Neurosci 9, 12-17.

Casado, M., Isope, P., and Ascher, P. (2002). Involvement of presynaptic N-methyl-D-aspartate receptors in cerebellar long-term depression. Neuron 33, 123-130.

Catania, M.V., Landwehrmeyer, G.B., Testa, C.M., Standaert, D.G., Penney, J.B., Jr., and Young, A.B. (1994). Metabotropic glutamate receptors are differentially regulated during development. Neuroscience 61, 481-495.

Catterall, W.A., Perez-Reyes, E., Snutch, T.P., and Striessnig, J. (2005). International Union of Pharmacology. XLVIII. Nomenclature and structure-function relationships of voltage-gated calcium channels. Pharmacol Rev 57, 411-425.

Cavelier, P., Beekenkamp, H., Shin, H.S., Jun, K., and Bossu, J.L. (2002a). Cerebellar slice cultures from mice lacking the P/Q calcium channel: electroresponsiveness of Purkinje cells. Neurosci Lett 333, 64-68.

Cavelier, P., and Bossu, J.L. (2003). Dendritic low-threshold $\mathrm{Ca}^{2+}$ channels in rat cerebellar Purkinje cells: possible physiological implications. Cerebellum 2, 196-205.

Cavelier, P., Desplantez, T., Beekenkamp, H., and Bossu, J.L. (2003). K+ channel activation and lowthreshold $\mathrm{Ca}^{2+}$ spike of rat cerebellar Purkinje cells in vitro. Neuroreport 14, 167-171.

Cavelier, P., Pouille, F., Desplantez, T., Beekenkamp, H., and Bossu, J.L. (2002b). Control of the propagation of dendritic low-threshold $\mathrm{Ca}^{2+}$ spikes in Purkinje cells from rat cerebellar slice cultures. J Physiol 540, 57-72.

Chemin, J., Mezghrani, A., Bidaud, I., Dupasquier, S., Marger, F., Barrere, C., Nargeot, J., and Lory, P. (2007a). Temperature-dependent modulation of CaV3 T-type calcium channels by protein kinases C and A in mammalian cells. J Biol Chem 282, 32710-32718.

Chemin, J., Monteil, A., Bourinet, E., Nargeot, J., and Lory, P. (2001a). Alternatively spliced alpha(1G) $(\mathrm{Ca}(\mathrm{V}) 3.1)$ intracellular loops promote specific T-type $\mathrm{Ca}(2+)$ channel gating properties. Biophys J 80 , 1238-1250.

Chemin, J., Monteil, A., Perez-Reyes, E., Bourinet, E., Nargeot, J., and Lory, P. (2002). Specific contribution of human T-type calcium channel isotypes (alpha ${ }_{1 \mathrm{G}}$, alpha $\mathrm{a}_{1 \mathrm{H}}$ and alpha ${ }_{1 \mathrm{I}}$ ) to neuronal excitability. J Physiol 540, 3-14.

Chemin, J., Monteil, A., Perez-Reyes, E., Nargeot, J., and Lory, P. (2001b). Direct inhibition of T-type calcium channels by the endogenous cannabinoid anandamide. Embo J 20, 7033-7040.

Chemin, J., Nargeot, J., and Lory, P. (2007b). Chemical determinants involved in anandamide-induced inhibition of T-type calcium channels. J Biol Chem 282, 2314-2323.

Chemin, J., Traboulsie, A., and Lory, P. (2006). Molecular pathways underlying the modulation of Ttype calcium channels by neurotransmitters and hormones. Cell Calcium 40, 121-134.

Chen, C., Xu, R., Clarke, I.J., Ruan, M., Loneragan, K., and Roh, S.G. (2000). Diverse intracellular signalling systems used by growth hormone-releasing hormone in regulating voltage-gated $\mathrm{Ca} 2+$ or K channels in pituitary somatotropes. Immunol Cell Biol 78, 356-368.

Chen, C.C., Lamping, K.G., Nuno, D.W., Barresi, R., Prouty, S.J., Lavoie, J.L., Cribbs, L.L., England, S.K., Sigmund, C.D., Weiss, R.M., et al. (2003a). Abnormal coronary function in mice deficient in alpha $_{1 H}$ T-type $\mathrm{Ca}^{2+}$ channels. Science 302, 1416-1418.

Chen, Y., Lu, J., Pan, H., Zhang, Y., Wu, H., Xu, K., Liu, X., Jiang, Y., Bao, X., Yao, Z., et al. (2003b). Association between genetic variation of CACNA1H and childhood absence epilepsy. Ann Neurol 54, 239-243.

Chen, Y., Sharp, A.H., Hata, K., Yunker, A.M., Polo-Parada, L., Landmesser, L.T., and McEnery, M.W. (2007). Site-directed antibodies to low-voltage-activated calcium channel CaV3.3 (alpha1I) subunit also target neural cell adhesion molecule-180. Neuroscience 145, 981-996.

Chevalier, M., Lory, P., Mironneau, C., Macrez, N., and Quignard, J.F. (2006). T-type CaV3.3 calcium channels produce spontaneous low-threshold action potentials and intracellular calcium oscillations. Eur J Neurosci 23, 2321-2329.

Christie, B.R., Schexnayder, L.K., and Johnston, D. (1997). Contribution of voltage-gated $\mathrm{Ca}^{2+}$ channels to homosynaptic long-term depression in the CA1 region in vitro. J Neurophysiol 77, 1651-1655.

Chuang, R.S., Jaffe, H., Cribbs, L., Perez-Reyes, E., and Swartz, K.J. (1998). Inhibition of T-type voltage-gated calcium channels by a new scorpion toxin. Nat Neurosci 1, 668-674.

Coesmans, M., Smitt, P.A., Linden, D.J., Shigemoto, R., Hirano, T., Yamakawa, Y., van Alphen, A.M., Luo, C., van der Geest, J.N., Kros, J.M., et al. (2003). Mechanisms underlying cerebellar motor deficits due to mGluR1-autoantibodies. Ann Neurol 53, 325-336.

Colwell, C.S., and Levine, M.S. (1999). Metabotropic glutamate receptor modulation of excitotoxicity in the neostriatum: role of calcium channels. Brain Res 833, 234-241.

Conn, P.J., and Pin, J.P. (1997). Pharmacology and functions of metabotropic glutamate receptors. Annu Rev Pharmacol Toxicol 37, 205-237.

Conquet, F., Bashir, Z.I., Davies, C.H., Daniel, H., Ferraguti, F., Bordi, F., Franz-Bacon, K., Reggiani, A., Matarese, V., Conde, F., and et al. (1994). Motor deficit and impairment of synaptic plasticity in mice lacking mGluR1. Nature 372, 237-243.

Coulter, D.A., Huguenard, J.R., and Prince, D.A. (1989). Calcium currents in rat thalamocortical relay neurones: kinetic properties of the transient, low-threshold current. J Physiol 414, 587-604.

Coutinho, V., and Knopfel, T. (2002). Metabotropic glutamate receptors: electrical and chemical signaling properties. Neuroscientist 8, 551-561.

Craig, P.J., Beattie, R.E., Folly, E.A., Banerjee, M.D., Reeves, M.B., Priestley, J.V., Carney, S.L., Sher, E., Perez-Reyes, E., and Volsen, S.G. (1999). Distribution of the voltage-dependent calcium channel alpha $_{1 \mathrm{G}}$ subunit mRNA and protein throughout the mature rat brain. Eur J Neurosci 11, 2949-2964.

Crepel, F., and Penit-Soria, J. (1986). Inward rectification and low threshold calcium conductance in rat cerebellar Purkinje cells. An in vitro study. J Physiol 372, 1-23.

Cribbs, L.L., Lee, J.H., Yang, J., Satin, J., Zhang, Y., Daud, A., Barclay, J., Williamson, M.P., Fox, M., Rees, M., and Perez-Reyes, E. (1998). Cloning and characterization of alpha ${ }_{1 H}$ from human heart, a member of the T-type $\mathrm{Ca}^{2+}$ channel gene family. Circ Res 83, 103-109.

Danthi, S.J., Enyeart, J.A., and Enyeart, J.J. (2005). Modulation of native T-type calcium channels by omega-3 fatty acids. Biochem Biophys Res Commun 327, 485-493.

Darszon, A., Lopez-Martinez, P., Acevedo, J.J., Hernandez-Cruz, A., and Trevino, C.L. (2006). T-type $\mathrm{Ca}^{2+}$ channels in sperm function. Cell Calcium 40, 241-252.

Dascal, N. (2001). Ion-channel regulation by G proteins. Trends Endocrinol Metab 12, 391-398.
De Zeeuw, C.I., Hansel, C., Bian, F., Koekkoek, S.K., van Alphen, A.M., Linden, D.J., and Oberdick, J. (1998). Expression of a protein kinase C inhibitor in Purkinje cells blocks cerebellar LTD and adaptation of the vestibulo-ocular reflex. Neuron 20, 495-508.

Denk, W., Sugimori, M., and Llinas, R. (1995). Two types of calcium response limited to single spines in cerebellar Purkinje cells. Proc Natl Acad Sci U S A 92, 8279-8282.

DePuy, S.D., Yao, J., Hu, C., McIntire, W., Bidaud, I., Lory, P., Rastinejad, F., Gonzalez, C., Garrison, J.C., and Barrett, P.Q. (2006). The molecular basis for T-type $\mathrm{Ca}^{2+}$ channel inhibition by G protein beta ${ }_{2}$ gamma ${ }_{2}$ subunits. Proc Natl Acad Sci U S A 103, 14590-14595.

Destexhe, A., Contreras, D., Steriade, M., Sejnowski, T.J., and Huguenard, J.R. (1996). In vivo, in vitro, and computational analysis of dendritic calcium currents in thalamic reticular neurons. J Neurosci 16, 169-185.

Destexhe, A., and Sejnowski, T.J. (2003). Interactions between membrane conductances underlying thalamocortical slow-wave oscillations. Physiol Rev 83, 1401-1453.

Diana, M.A., Otsu, Y., Maton, G., Collin, T., Chat, M., and Dieudonne, S. (2007). T-type and L-type $\mathrm{Ca}^{2+}$ conductances define and encode the bimodal firing pattern of vestibulocerebellar unipolar brush cells. J Neurosci 27, 3823-3838.

Dolphin, A.C. (2003). G protein modulation of voltage-gated calcium channels. Pharmacol Rev 55, 607627.

Dolphin, A.C. (2006). A short history of voltage-gated calcium channels. Br J Pharmacol 147 Suppl 1, S56-62.

Drolet, P., Bilodeau, L., Chorvatova, A., Laflamme, L., Gallo-Payet, N., and Payet, M.D. (1997). Inhibition of the T-type $\mathrm{Ca}^{2+}$ current by the dopamine D 1 receptor in rat adrenal glomerulosa cells: requirement of the combined action of the G betagamma protein subunit and cyclic adenosine $3^{\prime}, 5^{\prime}$ monophosphate. Mol Endocrinol 11, 503-514.

Dubel, S.J., Starr, T.V., Hell, J., Ahlijanian, M.K., Enyeart, J.J., Catterall, W.A., and Snutch, T.P. (1992). Molecular cloning of the alpha ${ }_{1}$ subunit of an omega-conotoxin-sensitive calcium channel. Proc Natl Acad Sci U S A 89, 5058-5062.

Dunlap, K., and Fischbach, G.D. (1978). Neurotransmitters decrease the calcium component of sensory neurone action potentials. Nature 276, 837-839.

Dunlap, K., and Fischbach, G.D. (1981). Neurotransmitters decrease the calcium conductance activated by depolarization of embryonic chick sensory neurones. J Physiol 317, 519-535.

Dzubay, J.A., and Otis, T.S. (2002). Climbing fiber activation of metabotropic glutamate receptors on cerebellar Purkinje neurons. Neuron 36, 1159-1167.

Edgerton, J.R., and Reinhart, P.H. (2003). Distinct contributions of small and large conductance $\mathrm{Ca}^{2+}$ activated K+ channels to rat Purkinje neuron function. J Physiol 548, 53-69.

Egger, V., Svoboda, K., and Mainen, Z.F. (2005). Dendrodendritic synaptic signals in olfactory bulb granule cells: local spine boost and global low-threshold spike. J Neurosci 25, 3521-3530.

Eilers, J., Augustine, G.J., and Konnerth, A. (1995). Subthreshold synaptic $\mathrm{Ca}^{2+}$ signalling in fine dendrites and spines of cerebellar Purkinje neurons. Nature 373, 155-158.

Eilers, J., Plant, T., and Konnerth, A. (1996). Localized calcium signalling and neuronal integration in cerebellar Purkinje neurones. Cell Calcium 20, 215-226.

Ekerot, C.F., and Jorntell, H. (2001). Parallel fibre receptive fields of Purkinje cells and interneurons are climbing fibre-specific. Eur J Neurosci 13, 1303-1310.

Ekerot, C.F., and Kano, M. (1985). Long-term depression of parallel fibre synapses following stimulation of climbing fibres. Brain Res 342, 357-360.

Elmslie, K.S. (2003). Neurotransmitter modulation of neuronal calcium channels. J Bioenerg Biomembr 35, 477-489.

Emerick, M.C., Stein, R., Kunze, R., McNulty, M.M., Regan, M.R., Hanck, D.A., and Agnew, W.S. (2006). Profiling the array of $\mathrm{Ca}_{\mathrm{v}} 3.1$ variants from the human T-type calcium channel gene CACNA1G: alternative structures, developmental expression, and biophysical variations. Proteins 64, 320-342.

Evans, G.J. (2007). Synaptic signalling in cerebellar plasticity. Biol Cell 99, 363-378.
Fatt, P., and Ginsborg, B.L. (1958). The ionic requirements for the production of action potentials in crustacean muscle fibres. J Physiol 142, 516-543.

Fatt, P., and Katz, B. (1953). The electrical properties of crustacean muscle fibres. J Physiol 120, 171204.

Finch, E.A., and Augustine, G.J. (1998). Local calcium signalling by inositol-1,4,5-trisphosphate in Purkinje cell dendrites. Nature 396, 753-756.

Fisher, R., and Johnston, D. (1990). Differential modulation of single voltage-gated calcium channels by cholinergic and adrenergic agonists in adult hippocampal neurons. J Neurophysiol 64, 1291-1302.

Fleckenstein, A. (1983). History of calcium antagonists. Circ Res 52, I3-16.
Flucher, B.E., and Franzini-Armstrong, C. (1996). Formation of junctions involved in excitationcontraction coupling in skeletal and cardiac muscle. Proc Natl Acad Sci U S A 93, 8101-8106.

Formenti, A., and Sansone, V. (1991). Inhibitory action of acetylcholine, baclofen and GTP-gamma-S on calcium channels in adult rat sensory neurons. Neurosci Lett 131, 267-272.

Fox, A.P., Nowycky, M.C., and Tsien, R.W. (1987a). Kinetic and pharmacological properties distinguishing three types of calcium currents in chick sensory neurones. J Physiol 394, 149-172.

Fox, A.P., Nowycky, M.C., and Tsien, R.W. (1987b). Single-channel recordings of three types of calcium channels in chick sensory neurones. J Physiol 394, 173-200.

Fraser, D.D., and MacVicar, B.A. (1991). Low-threshold transient calcium current in rat hippocampal lacunosum-moleculare interneurons: kinetics and modulation by neurotransmitters. J Neurosci 11, 28122820.

Freeze, B.S., McNulty, M.M., and Hanck, D.A. (2006). State-dependent verapamil block of the cloned human $\mathrm{Ca}(\mathrm{v}) 3.1$ T-type $\mathrm{Ca}(2+)$ channel. Mol Pharmacol 70, 718-726.

Gamper, N., Reznikov, V., Yamada, Y., Yang, J., and Shapiro, M.S. (2004). Phosphatidylinositol-4,5bisphosphate signals underlie receptor-specific $\mathrm{G}_{\mathrm{q} / 11}-$ mediated modulation of N-type $\mathrm{Ca}^{2+}$ channels. J Neurosci 24, 10980-10992.

Gamper, N., and Shapiro, M.S. (2007). Regulation of ion transport proteins by membrane phosphoinositides. Nat Rev Neurosci 8, 921-934.

Gao, W., Dunbar, R.L., Chen, G., Reinert, K.C., Oberdick, J., and Ebner, T.J. (2003). Optical imaging of long-term depression in the mouse cerebellar cortex in vivo. J Neurosci 23, 1859-1866.

Ghez, C., and Thach, W.T. (2000). The cerebellum. In Principles of Neural Science, E.R. Kandel, J.H. Schwartz, and T.M. Jessell, eds. (New York, NY: McGraw-Hill Companies), pp. 832-852.

Golding, N.L., Staff, N.P., and Spruston, N. (2002). Dendritic spikes as a mechanism for cooperative long-term potentiation. Nature 418, 326-331.

Gomora, J.C., Daud, A.N., Weiergraber, M., and Perez-Reyes, E. (2001). Block of cloned human T-type calcium channels by succinimide antiepileptic drugs. Mol Pharmacol 60, 1121-1132.

Gomora, J.C., Murbartian, J., Arias, J.M., Lee, J.H., and Perez-Reyes, E. (2002). Cloning and expression of the human T-type channel $\mathrm{Ca}_{\mathrm{v}} 3.3$ : insights into prepulse facilitation. Biophys J 83, 229-241.

Gruol, D.L., Deal, C.R., and Yool, A.J. (1992). Developmental changes in calcium conductances contribute to the physiological maturation of cerebellar Purkinje neurons in culture. J Neurosci 12, 28382848.

Hagiwara, S., Ozawa, S., and Sand, O. (1975). Voltage clamp analysis of two inward current mechanisms in the egg cell membrane of a starfish. J Gen Physiol 65, 617-644.

Hamid, J., Nelson, D., Spaetgens, R., Dubel, S.J., Snutch, T.P., and Zamponi, G.W. (1999). Identification of an integration center for cross-talk between protein kinase C and G protein modulation of N-type calcium channels. J Biol Chem 274, 6195-6202.

Hamid, J., Peloquin, J.B., Monteil, A., and Zamponi, G.W. (2006). Determinants of the differential gating properties of Cav3.1 and Cav3.3 T-type channels: a role of domain IV? Neuroscience 143, 717728.

Harvey, R.J., and Napper, R.M. (1991). Quantitative studies on the mammalian cerebellum. Prog Neurobiol 36, 437-463.

Hashimoto, K., and Kano, M. (2005). Postnatal development and synapse elimination of climbing fiber to Purkinje cell projection in the cerebellum. Neurosci Res 53, 221-228.

Hayashi, K., Wakino, S., Sugano, N., Ozawa, Y., Homma, K., and Saruta, T. (2007). Ca2+ channel subtypes and pharmacology in the kidney. Circ Res 100, 342-353.

Hayashi, Y., Tanabe, Y., Aramori, I., Masu, M., Shimamoto, K., Ohfune, Y., and Nakanishi, S. (1992). Agonist analysis of 2-(carboxycyclopropyl)glycine isomers for cloned metabotropic glutamate receptor subtypes expressed in Chinese hamster ovary cells. Br J Pharmacol 107, 539-543.

Hell, J.W., Westenbroek, R.E., Warner, C., Ahlijanian, M.K., Prystay, W., Gilbert, M.M., Snutch, T.P., and Catterall, W.A. (1993). Identification and differential subcellular localization of the neuronal class C and class D L-type calcium channel alpha 1 subunits. J Cell Biol 123, 949-962.

Herlitze, S., Garcia, D.E., Mackie, K., Hille, B., Scheuer, T., and Catterall, W.A. (1996). Modulation of $\mathrm{Ca} 2+$ channels by G-protein beta gamma subunits. Nature 380, 258-262.

Heron, S.E., Khosravani, H., Varela, D., Bladen, C., Williams, T.C., Newman, M.R., Scheffer, I.E., Berkovic, S.F., Mulley, J.C., and Zamponi, G.W. (2007). Extended spectrum of idiopathic generalized epilepsies associated with CACNA1H functional variants. Ann Neurol 62, 560-568.

Hildebrand, M.E., and Snutch, T.P. (2006). Contributions of T-type calcium channels to the pathophysiology of pain signaling. Drug Discovery Today: Disease Mechanisms 3, 335-341.

Hirano, T., and Hagiwara, S. (1989). Kinetics and distribution of voltage-gated $\mathrm{Ca}, \mathrm{Na}$ and K channels on the somata of rat cerebellar Purkinje cells. Pflugers Arch 413, 463-469.

Hirono, M., Konishi, S., and Yoshioka, T. (1998). Phospholipase C-independent group I metabotropic glutamate receptor-mediated inward current in mouse purkinje cells. Biochem Biophys Res Commun 251, 753-758.

Houamed, K.M., Kuijper, J.L., Gilbert, T.L., Haldeman, B.A., O'Hara, P.J., Mulvihill, E.R., Almers, W., and Hagen, F.S. (1991). Cloning, expression, and gene structure of a G protein-coupled glutamate receptor from rat brain. Science 252, 1318-1321.

Howe, A.R., and Surmeier, D.J. (1995). Muscarinic receptors modulate N-, P-, and L-type Ca2+ currents in rat striatal neurons through parallel pathways. J Neurosci 15, 458-469.

Huguenard, J.R. (1996). Low-threshold calcium currents in central nervous system neurons. Annu Rev Physiol 58, 329-348.

Huguenard, J.R., and Prince, D.A. (1992). A novel T-type current underlies prolonged $\mathrm{Ca}(2+$ )-dependent burst firing in GABAergic neurons of rat thalamic reticular nucleus. J Neurosci 12, 3804-3817.

Huguenard, J.R., and Prince, D.A. (1994). Intrathalamic rhythmicity studied in vitro: nominal T-current modulation causes robust antioscillatory effects. J Neurosci 14, 5485-5502.

Ichise, T., Kano, M., Hashimoto, K., Yanagihara, D., Nakao, K., Shigemoto, R., Katsuki, M., and Aiba, A. (2000). mGluR1 in cerebellar Purkinje cells essential for long-term depression, synapse elimination, and motor coordination. Science 288, 1832-1835.

Iftinca, M., McKay, B.E., Snutch, T.P., McRory, J.E., Turner, R.W., and Zamponi, G.W. (2006). Temperature dependence of T-type calcium channel gating. Neuroscience 142, 1031-1042.

Ikeda, H., Heinke, B., Ruscheweyh, R., and Sandkuhler, J. (2003). Synaptic plasticity in spinal lamina I projection neurons that mediate hyperalgesia. Science 299, 1237-1240.

Ikeda, S.R. (1996). Voltage-dependent modulation of N-type calcium channels by G-protein beta gamma subunits. Nature 380, 255-258.

Inoue, T. (2003). Dynamics of calcium and its roles in the dendrite of the cerebellar Purkinje cell. Keio J Med 52, 244-249.

Ishii, M., and Kurachi, Y. (2006). Muscarinic acetylcholine receptors. Curr Pharm Des 12, 3573-3581.
Isope, P., and Barbour, B. (2002). Properties of unitary granule cell-->Purkinje cell synapses in adult rat cerebellar slices. J Neurosci 22, 9668-9678.

Isope, P., and Murphy, T.H. (2005). Low threshold calcium currents in rat cerebellar Purkinje cell dendritic spines are mediated by T-type calcium channels. J Physiol 562, 257-269.

Ito, M. (2001). Cerebellar long-term depression: characterization, signal transduction, and functional roles. Physiol Rev 81, 1143-1195.

Ito, M. (2002). The molecular organization of cerebellar long-term depression. Nat Rev Neurosci 3, 896902.

Ito, M., Sakurai, M., and Tongroach, P. (1982). Climbing fibre induced depression of both mossy fibre responsiveness and glutamate sensitivity of cerebellar Purkinje cells. J Physiol 324, 113-134.

Joksovic, P.M., Bayliss, D.A., and Todorovic, S.M. (2005). Different kinetic properties of two T-type $\mathrm{Ca} 2+$ currents of rat reticular thalamic neurones and their modulation by enflurane. J Physiol 566, 125142.

Joksovic, P.M., Nelson, M.T., Jevtovic-Todorovic, V., Patel, M.K., Perez-Reyes, E., Campbell, K.P., Chen, C.C., and Todorovic, S.M. (2006). CaV3.2 is the major molecular substrate for redox regulation of T-type Ca2+ channels in the rat and mouse thalamus. J Physiol 574, 415-430.

Kammermeier, P.J., and Ikeda, S.R. (1999). Expression of RGS2 alters the coupling of metabotropic glutamate receptor 1a to M-type K+ and N-type Ca2+ channels. Neuron 22, 819-829.

Kammermeier, P.J., Ruiz-Velasco, V., and Ikeda, S.R. (2000). A voltage-independent calcium current inhibitory pathway activated by muscarinic agonists in rat sympathetic neurons requires both Galpha $\mathrm{q} / 11$ and Gbeta gamma. J Neurosci 20, 5623-5629.

Kaneda, M., Wakamori, M., Ito, C., and Akaike, N. (1990). Low-threshold calcium current in isolated Purkinje cell bodies of rat cerebellum. J Neurophysiol 63, 1046-1051.

Kang, H.W., Park, J.Y., Jeong, S.W., Kim, J.A., Moon, H.J., Perez-Reyes, E., and Lee, J.H. (2006). A molecular determinant of nickel inhibition in Cav3.2 T-type calcium channels. J Biol Chem 281, 48234830.

Kano, M., Hashimoto, K., Kurihara, H., Watanabe, M., Inoue, Y., Aiba, A., and Tonegawa, S. (1997). Persistent multiple climbing fiber innervation of cerebellar Purkinje cells in mice lacking mGluR1. Neuron 18, 71-79.

Kase, M., Kakimoto, S., Sakuma, S., Houtani, T., Ohishi, H., Ueyama, T., and Sugimoto, T. (1999). Distribution of neurons expressing alpha 1G subunit mRNA of T-type voltage-dependent calcium channel in adult rat central nervous system. Neurosci Lett 268, 77-80.

Katz, B., and Miledi, R. (1970). Further study of the role of calcium in synaptic transmission. J Physiol 207, 789-801.

Kavalali, E.T., Zhuo, M., Bito, H., and Tsien, R.W. (1997). Dendritic Ca2+ channels characterized by recordings from isolated hippocampal dendritic segments. Neuron 18, 651-663.

Khosravani, H., Altier, C., Simms, B., Hamming, K.S., Snutch, T.P., Mezeyova, J., McRory, J.E., and Zamponi, G.W. (2004). Gating effects of mutations in the Cav3.2 T-type calcium channel associated with childhood absence epilepsy. J Biol Chem 279, 9681-9684.

Khosravani, H., Bladen, C., Parker, D.B., Snutch, T.P., McRory, J.E., and Zamponi, G.W. (2005). Effects of $\mathrm{Ca}(\mathrm{v}) 3.2$ channel mutations linked to idiopathic generalized epilepsy. Ann Neurol 57, 745749.

Khosravani, H., and Zamponi, G.W. (2006). Voltage-gated calcium channels and idiopathic generalized epilepsies. Physiol Rev 86, 941-966.

Kim, D., Song, I., Keum, S., Lee, T., Jeong, M.J., Kim, S.S., McEnery, M.W., and Shin, H.S. (2001). Lack of the burst firing of thalamocortical relay neurons and resistance to absence seizures in mice lacking alpha(1G) T-type $\mathrm{Ca}(2+$ ) channels. Neuron 31, 35-45.

Kim, H.S., Kim, Y., Doddareddy, M.R., Seo, S.H., Rhim, H., Tae, J., Pae, A.N., Choo, H., and Cho, Y.S. (2007). Design, synthesis, and biological evaluation of 1,3-dioxoisoindoline-5-carboxamide derivatives as T-type calcium channel blockers. Bioorg Med Chem Lett 17, 476-481.

Kim, J.A., Park, J.Y., Kang, H.W., Huh, S.U., Jeong, S.W., and Lee, J.H. (2006). Augmentation of Cav3.2 T-type calcium channel activity by cAMP-dependent protein kinase A. J Pharmacol Exp Ther 318, 230-237.

Kim, S.J., Kim, Y.S., Yuan, J.P., Petralia, R.S., Worley, P.F., and Linden, D.J. (2003). Activation of the TRPC1 cation channel by metabotropic glutamate receptor mGluR1. Nature 426, 285-291.

Kishimoto, Y., Fujimichi, R., Araishi, K., Kawahara, S., Kano, M., Aiba, A., and Kirino, Y. (2002). mGluR1 in cerebellar Purkinje cells is required for normal association of temporally contiguous stimuli in classical conditioning. Eur J Neurosci 16, 2416-2424.

Kitano, J., Nishida, M., Itsukaichi, Y., Minami, I., Ogawa, M., Hirano, T., Mori, Y., and Nakanishi, S. (2003). Direct interaction and functional coupling between metabotropic glutamate receptor subtype 1 and voltage-sensitive Cav2.1 Ca2+ channel. J Biol Chem 278, 25101-25108.

Klockner, U., Lee, J.H., Cribbs, L.L., Daud, A., Hescheler, J., Pereverzev, A., Perez-Reyes, E., and Schneider, T. (1999). Comparison of the Ca2 + currents induced by expression of three cloned alpha1 subunits, alpha1G, alpha1H and alpha1I, of low-voltage-activated T-type Ca2 + channels. Eur J Neurosci 11, 4171-4178.

Knopfel, T., and Grandes, P. (2002). Metabotropic glutamate receptors in the cerebellum with a focus on their function in Purkinje cells. Cerebellum 1, 19-26.

Knopfel, T., Vranesic, I., Staub, C., and Gahwiler, B.H. (1991). Climbing Fibre Responses in Olivocerebellar Slice Cultures. II. Dynamics of Cytosolic Calcium in Purkinje Cells. Eur J Neurosci 3, 343348.

Kraus, R.L., Li, Y., Jovanovska, A., and Renger, J.J. (2007). Trazodone inhibits T-type calcium channels. Neuropharmacology 53, 308-317.

Kumar, P.P., Stotz, S.C., Paramashivappa, R., Beedle, A.M., Zamponi, G.W., and Rao, A.S. (2002). Synthesis and evaluation of a new class of nifedipine analogs with T-type calcium channel blocking activity. Mol Pharmacol 61, 649-658.

Kurejova, M., and Lacinova, L. (2006). Effect of protein tyrosine kinase inhibitors on the current through the $\mathrm{Ca}(\mathrm{V}) 3.1$ channel. Arch Biochem Biophys 446, 20-27.

Kurejova, M., Lacinova, L., Pavlovicova, M., Eschbach, M., and Klugbauer, N. (2007). The effect of the outermost basic residues in the S 4 segments of the $\mathrm{Ca}(\mathrm{V}) 3.1$ T-type calcium channel on channel gating. Pflugers Arch 455, 527-539.

Lam, A.D., Chikina, M.D., McNulty, M.M., Glaaser, I.W., and Hanck, D.A. (2005). Role of Domain IV/S4 outermost arginines in gating of T-type calcium channels. Pflugers Arch 451, 349-361.

Lanzafame, A.A., Christopoulos, A., and Mitchelson, F. (2003). Cellular signaling mechanisms for muscarinic acetylcholine receptors. Receptors Channels 9, 241-260.

Lee, A., and Catterall, W.A. (2005). $\mathrm{Ca}^{2+}$-dependent modulation of voltage-gated $\mathrm{Ca}^{2+}$ channels. In Voltage-gated calcium channels, G.W. Zamponi, ed. (New York: Landes Bioscience), pp. 183-193.

Lee, A., Wong, S.T., Gallagher, D., Li, B., Storm, D.R., Scheuer, T., and Catterall, W.A. (1999a). $\mathrm{Ca} 2+/$ calmodulin binds to and modulates P/Q-type calcium channels. Nature 399, 155-159.

Lee, J.H., Daud, A.N., Cribbs, L.L., Lacerda, A.E., Pereverzev, A., Klockner, U., Schneider, T., and Perez-Reyes, E. (1999b). Cloning and expression of a novel member of the low voltage-activated T-type calcium channel family. J Neurosci 19, 1912-1921.

Lee, J.H., Gomora, J.C., Cribbs, L.L., and Perez-Reyes, E. (1999c). Nickel block of three cloned T-type calcium channels: low concentrations selectively block alpha1H. Biophys J 77, 3034-3042.

Li, J., Stevens, L., Klugbauer, N., and Wray, D. (2004). Roles of molecular regions in determining differences between voltage dependence of activation of CaV 3.1 and CaV 1.2 calcium channels. J Biol Chem 279, 26858-26867.

Li, L., Bischofberger, J., and Jonas, P. (2007). Differential gating and recruitment of P/Q-, N-, and Rtype Ca2+ channels in hippocampal mossy fiber boutons. J Neurosci 27, 13420-13429.

Liang, J., Zhang, Y., Chen, Y., Wang, J., Pan, H., Wu, H., Xu, K., Liu, X., Jiang, Y., Shen, Y., and Wu, X. (2007). Common polymorphisms in the CACNA1H gene associated with childhood absence epilepsy in Chinese Han population. Ann Hum Genet 71, 325-335.

Lin, F.F., Varney, M., Sacaan, A.I., Jachec, C., Daggett, L.P., Rao, S., Flor, P., Kuhn, R., Kerner, J.A., Standaert, D., et al. (1997). Cloning and stable expression of the mGluR1b subtype of human metabotropic receptors and pharmacological comparison with the mGluR5a subtype. Neuropharmacology 36, 917-931.

Linden, D.J. (2001). The expression of cerebellar LTD in culture is not associated with changes in AMPA-receptor kinetics, agonist affinity, or unitary conductance. Proc Natl Acad Sci U S A 98, 1406614071.

Linden, D.J., and Connor, J.A. (1991). Participation of postsynaptic PKC in cerebellar long-term depression in culture. Science 254, 1656-1659.

Lipscombe, D., Helton, T.D., and Xu, W. (2004). L-type calcium channels: the low down. J Neurophysiol 92, 2633-2641.

Lipscombe, D., Kongsamut, S., and Tsien, R.W. (1989). Alpha-adrenergic inhibition of sympathetic neurotransmitter release mediated by modulation of N-type calcium-channel gating. Nature 340, 639642.

Liu, L., and Rittenhouse, A.R. (2003). Arachidonic acid mediates muscarinic inhibition and enhancement of N-type Ca2+ current in sympathetic neurons. Proc Natl Acad Sci U S A 100, 295-300.

Liu, L., Zhao, R., Bai, Y., Stanish, L.F., Evans, J.E., Sanderson, M.J., Bonventre, J.V., and Rittenhouse, A.R. (2006). M1 muscarinic receptors inhibit L-type Ca2+ current and M-current by divergent signal transduction cascades. J Neurosci 26, 11588-11598.

Llinas, R., Steinberg, I.Z., and Walton, K. (1976). Presynaptic calcium currents and their relation to synaptic transmission: voltage clamp study in squid giant synapse and theoretical model for the calcium gate. Proc Natl Acad Sci U S A 73, 2918-2922.

Llinas, R., and Sugimori, M. (1980a). Electrophysiological properties of in vitro Purkinje cell dendrites in mammalian cerebellar slices. J Physiol 305, 197-213.

Llinas, R., and Sugimori, M. (1980b). Electrophysiological properties of in vitro Purkinje cell somata in mammalian cerebellar slices. J Physiol 305, 171-195.

Llinas, R., and Yarom, Y. (1981). Electrophysiology of mammalian inferior olivary neurones in vitro. Different types of voltage-dependent ionic conductances. J Physiol 315, 549-567.

Loewenstein, Y., Mahon, S., Chadderton, P., Kitamura, K., Sompolinsky, H., Yarom, Y., and Hausser, M. (2005). Bistability of cerebellar Purkinje cells modulated by sensory stimulation. Nat Neurosci 8 , 202-211.

Lopez-Bendito, G., Shigemoto, R., Lujan, R., and Juiz, J.M. (2001). Developmental changes in the localisation of the mGluR 1alpha subtype of metabotropic glutamate receptors in Purkinje cells. Neuroscience 105, 413-429.

Lu, H.K., Fern, R.J., Nee, J.J., and Barrett, P.Q. (1994). Ca(2+)-dependent activation of T-type Ca2+ channels by calmodulin-dependent protein kinase II. Am J Physiol 267, F183-189.

Maeda, H., Ellis-Davies, G.C., Ito, K., Miyashita, Y., and Kasai, H. (1999). Supralinear Ca2+ signaling by cooperative and mobile Ca2+ buffering in Purkinje neurons. Neuron 24, 989-1002.

Magee, J.C., Avery, R.B., Christie, B.R., and Johnston, D. (1996). Dihydropyridine-sensitive, voltagegated $\mathrm{Ca} 2+$ channels contribute to the resting intracellular Ca2+ concentration of hippocampal CA1 pyramidal neurons. J Neurophysiol 76, 3460-3470.

Magee, J.C., Christofi, G., Miyakawa, H., Christie, B., Lasser-Ross, N., and Johnston, D. (1995). Subthreshold synaptic activation of voltage-gated $\mathrm{Ca} 2+$ channels mediates a localized $\mathrm{Ca} 2+$ influx into the dendrites of hippocampal pyramidal neurons. J Neurophysiol 74, 1335-1342.

Markram, H., and Sakmann, B. (1994). Calcium transients in dendrites of neocortical neurons evoked by single subthreshold excitatory postsynaptic potentials via low-voltage-activated calcium channels. Proc Natl Acad Sci U S A 91, 5207-5211.

Marksteiner, R., Schurr, P., Berjukow, S., Margreiter, E., Perez-Reyes, E., and Hering, S. (2001). Inactivation determinants in segment IIIS6 of Ca(v)3.1. J Physiol 537, 27-34.

Massie, B.M. (1997). Mibefradil: a selective T-type calcium antagonist. Am J Cardiol 80, 23I-32I.
Masu, M., Tanabe, Y., Tsuchida, K., Shigemoto, R., and Nakanishi, S. (1991). Sequence and expression of a metabotropic glutamate receptor. Nature 349, 760-765.

Mateos, J.M., Benitez, R., Elezgarai, I., Azkue, J.J., Lazaro, E., Osorio, A., Bilbao, A., Donate, F., Sarria, R., Conquet, F., et al. (2000). Immunolocalization of the mGluR1b splice variant of the metabotropic glutamate receptor 1 at parallel fiber-Purkinje cell synapses in the rat cerebellar cortex. J Neurochem 74, 1301-1309.

McCool, B.A., Pin, J.P., Brust, P.F., Harpold, M.M., and Lovinger, D.M. (1996). Functional coupling of rat group II metabotropic glutamate receptors to an omega-conotoxin GVIA-sensitive calcium channel in human embryonic kidney 293 cells. Mol Pharmacol 50, 912-922.

McCool, B.A., Pin, J.P., Harpold, M.M., Brust, P.F., Stauderman, K.A., and Lovinger, D.M. (1998). Rat group I metabotropic glutamate receptors inhibit neuronal $\mathrm{Ca} 2+$ channels via multiple signal transduction pathways in HEK 293 cells. J Neurophysiol 79, 379-391.

McCormick, D.A., and Contreras, D. (2001). On the cellular and network bases of epileptic seizures. Annu Rev Physiol 63, 815-846.

McDonough, S.I., and Bean, B.P. (1998). Mibefradil inhibition of T-type calcium channels in cerebellar purkinje neurons. Mol Pharmacol 54, 1080-1087.

McKay, B.E., Engbers, J.D., Mehaffey, W.H., Gordon, G.R., Molineux, M.L., Bains, J.S., and Turner, R.W. (2007). Climbing fiber discharge regulates cerebellar functions by controlling the intrinsic characteristics of purkinje cell output. J Neurophysiol 97, 2590-2604.

McKay, B.E., McRory, J.E., Molineux, M.L., Hamid, J., Snutch, T.P., Zamponi, G.W., and Turner, R.W. (2006). $\mathrm{Ca}(\mathrm{V}) 3$ T-type calcium channel isoforms differentially distribute to somatic and dendritic compartments in rat central neurons. Eur J Neurosci 24, 2581-2594.

McRory, J.E., Hamid, J., Doering, C.J., Garcia, E., Parker, R., Hamming, K., Chen, L., Hildebrand, M., Beedle, A.M., Feldcamp, L., et al. (2004). The CACNA1F gene encodes an L-type calcium channel with unique biophysical properties and tissue distribution. J Neurosci 24, 1707-1718.

McRory, J.E., Santi, C.M., Hamming, K.S., Mezeyova, J., Sutton, K.G., Baillie, D.L., Stea, A., and Snutch, T.P. (2001). Molecular and functional characterization of a family of rat brain T-type calcium channels. J Biol Chem 276, 3999-4011.

Melliti, K., Meza, U., and Adams, B. (2000). Muscarinic stimulation of alpha1E Ca channels is selectively blocked by the effector antagonist function of RGS2 and phospholipase C-beta1. J Neurosci 20, 7167-7173.

Melliti, K., Meza, U., and Adams, B.A. (2001). RGS2 blocks slow muscarinic inhibition of N-type $\mathrm{Ca}(2+)$ channels reconstituted in a human cell line. J Physiol 532, 337-347.

Mintz, I.M., Adams, M.E., and Bean, B.P. (1992). P-type calcium channels in rat central and peripheral neurons. Neuron 9, 85-95.

Mittman, S., Guo, J., Emerick, M.C., and Agnew, W.S. (1999). Structure and alternative splicing of the gene encoding alpha1I, a human brain T calcium channel alpha1 subunit. Neurosci Lett 269, 121-124.

Miyakawa, H., Lev-Ram, V., Lasser-Ross, N., and Ross, W.N. (1992). Calcium transients evoked by climbing fiber and parallel fiber synaptic inputs in guinea pig cerebellar Purkinje neurons. J Neurophysiol 68, 1178-1189.

Miyata, M., Finch, E.A., Khiroug, L., Hashimoto, K., Hayasaka, S., Oda, S.I., Inouye, M., Takagishi, Y., Augustine, G.J., and Kano, M. (2000). Local calcium release in dendritic spines required for long-term synaptic depression. Neuron 28, 233-244.

Molineux, M.L., McRory, J.E., McKay, B.E., Hamid, J., Mehaffey, W.H., Rehak, R., Snutch, T.P., Zamponi, G.W., and Turner, R.W. (2006). Specific T-type calcium channel isoforms are associated with distinct burst phenotypes in deep cerebellar nuclear neurons. Proc Natl Acad Sci U S A 103, 5555-5560.

Monteil, A., Chemin, J., Bourinet, E., Mennessier, G., Lory, P., and Nargeot, J. (2000). Molecular and functional properties of the human alpha $(1 \mathrm{G})$ subunit that forms T-type calcium channels. J Biol Chem 275, 6090-6100.

Morikawa, H., Fukuda, K., Mima, H., Shoda, T., Kato, S., and Mori, K. (1998). Tyrosine kinase inhibitors suppress N-type and T-type Ca2+ channel currents in NG108-15 cells. Pflugers Arch 436, 127-132.

Mouginot, D., Bossu, J.L., and Gahwiler, B.H. (1997). Low-threshold Ca2+ currents in dendritic recordings from Purkinje cells in rat cerebellar slice cultures. J Neurosci 17, 160-170.

Murbartian, J., Arias, J.M., and Perez-Reyes, E. (2004). Functional impact of alternative splicing of human T-type Cav3.3 calcium channels. J Neurophysiol 92, 3399-3407.

Nakanishi, S. (1992). Molecular diversity of glutamate receptors and implications for brain function. Science 258, 597-603.

Narasimhan, K., Pessah, I.N., and Linden, D.J. (1998). Inositol-1,4,5-trisphosphate receptor-mediated Ca mobilization is not required for cerebellar long-term depression in reduced preparations. J Neurophysiol 80, 2963-2974.

Nelson, M.T., Joksovic, P.M., Perez-Reyes, E., and Todorovic, S.M. (2005). The endogenous redox agent L-cysteine induces T-type Ca2+ channel-dependent sensitization of a novel subpopulation of rat peripheral nociceptors. J Neurosci 25, 8766-8775.

Nelson, M.T., Joksovic, P.M., Su, P., Kang, H.W., Van Deusen, A., Baumgart, J.P., David, L.S., Snutch, T.P., Barrett, P.Q., Lee, J.H., et al. (2007a). Molecular mechanisms of subtype-specific inhibition of neuronal T-type calcium channels by ascorbate. J Neurosci 27, 12577-12583.

Nelson, M.T., Woo, J., Kang, H.W., Vitko, I., Barrett, P.Q., Perez-Reyes, E., Lee, J.H., Shin, H.S., and Todorovic, S.M. (2007b). Reducing agents sensitize C-type nociceptors by relieving high-affinity zinc inhibition of T-type calcium channels. J Neurosci 27, 8250-8260.

Novara, M., Baldelli, P., Cavallari, D., Carabelli, V., Giancippoli, A., and Carbone, E. (2004). Exposure to cAMP and beta-adrenergic stimulation recruits $\mathrm{Ca}(\mathrm{V}) 3$ T-type channels in rat chromaffin cells through Epac cAMP-receptor proteins. J Physiol 558, 433-449.

Park, J.Y., Kang, H.W., Jeong, S.W., and Lee, J.H. (2004). Multiple structural elements contribute to the slow kinetics of the Cav3.3 T-type channel. J Biol Chem 279, 21707-21713.

Park, J.Y., Kang, H.W., Moon, H.J., Huh, S.U., Jeong, S.W., Soldatov, N.M., and Lee, J.H. (2006). Activation of protein kinase C augments T-type Ca2+ channel activity without changing channel surface density. J Physiol 577, 513-523.

Peloquin, J.B., Khosravani, H., Barr, W., Bladen, C., Evans, R., Mezeyova, J., Parker, D., Snutch, T.P., McRory, J.E., and Zamponi, G.W. (2006). Functional analysis of Ca3.2 T-type calcium channel mutations linked to childhood absence epilepsy. Epilepsia 47, 655-658.

Pemberton, K.E., Hill-Eubanks, L.J., and Jones, S.V. (2000). Modulation of low-threshold T-type calcium channels by the five muscarinic receptor subtypes in NIH 3 T3 cells. Pflugers Arch 440, 452461.

Pemberton, K.E., and Jones, S.V. (1997). Inhibition of the L-type calcium channel by the five muscarinic receptors (m1-m5) expressed in NIH 3T3 cells. Pflugers Arch 433, 505-514.

Perez-Reyes, E. (2003). Molecular physiology of low-voltage-activated t-type calcium channels. Physiol Rev 83, 117-161.

Perez-Reyes, E., Cribbs, L.L., Daud, A., Lacerda, A.E., Barclay, J., Williamson, M.P., Fox, M., Rees, M., and Lee, J.H. (1998). Molecular characterization of a neuronal low-voltage-activated T-type calcium channel. Nature 391, 896-900.

Pouille, F., Cavelier, P., Desplantez, T., Beekenkamp, H., Craig, P.J., Beattie, R.E., Volsen, S.G., and Bossu, J.L. (2000). Dendro-somatic distribution of calcium-mediated electrogenesis in purkinje cells from rat cerebellar slice cultures. J Physiol 527 Pt 2, 265-282.

Raingo, J., Castiglioni, A.J., and Lipscombe, D. (2007). Alternative splicing controls G proteindependent inhibition of N-type calcium channels in nociceptors. Nat Neurosci 10, 285-292.

Raman, I.M., and Bean, B.P. (1999). Ionic currents underlying spontaneous action potentials in isolated cerebellar Purkinje neurons. J Neurosci 19, 1663-1674.

Randall, A.D., and Tsien, R.W. (1997). Contrasting biophysical and pharmacological properties of Ttype and R-type calcium channels. Neuropharmacology 36, 879-893.

Reuter, H. (1967). The dependence of slow inward current in Purkinje fibres on the extracellular calcium-concentration. J Physiol 192, 479-492.

Rhim, H., Lee, Y.S., Park, S.J., Chung, B.Y., and Lee, J.Y. (2005). Synthesis and biological activity of 3,4-dihydroquinazolines for selective T-type Ca2+ channel blockers. Bioorg Med Chem Lett 15, 283286.

Robbins, J., Reynolds, A.M., Treseder, S., and Davies, R. (2003). Enhancement of low-voltage-activated calcium currents by group II metabotropic glutamate receptors in rat retinal ganglion cells. Mol Cell Neurosci 23, 341-350.

Rosati, B., Dun, W., Hirose, M., Boyden, P.A., and McKinnon, D. (2007). Molecular basis of the T- and L-type Ca2+ currents in canine Purkinje fibres. J Physiol 579, 465-471.

Rossier, M.F., Aptel, H.B., Python, C.P., Burnay, M.M., Vallotton, M.B., and Capponi, A.M. (1995). Inhibition of low threshold calcium channels by angiotensin II in adrenal glomerulosa cells through activation of protein kinase C. J Biol Chem 270, 15137-15142.

Roth, A., and Hausser, M. (2001). Compartmental models of rat cerebellar Purkinje cells based on simultaneous somatic and dendritic patch-clamp recordings. J Physiol 535, 445-472.

Santi, C.M., Cayabyab, F.S., Sutton, K.G., McRory, J.E., Mezeyova, J., Hamming, K.S., Parker, D., Stea, A., and Snutch, T.P. (2002). Differential inhibition of T-type calcium channels by neuroleptics. J Neurosci 22, 396-403.

Satoh, R., Nakabayashi, Y., and Kano, M. (1991). Pharmacological properties of two types of calcium channel in embryonic chick skeletal muscle cells in culture. Neurosci Lett 122, 233-236.

Schmidt-Hieber, C., Jonas, P., and Bischofberger, J. (2004). Enhanced synaptic plasticity in newly generated granule cells of the adult hippocampus. Nature 429, 184-187.

Schmidt, H., Brown, E.B., Schwaller, B., and Eilers, J. (2003). Diffusional mobility of parvalbumin in spiny dendrites of cerebellar Purkinje neurons quantified by fluorescence recovery after photobleaching. Biophys J 84, 2599-2608.

Schmolesky, M.T., Weber, J.T., De Zeeuw, C.I., and Hansel, C. (2002). The making of a complex spike: ionic composition and plasticity. Ann N Y Acad Sci 978, 359-390.

Schoepp, D.D., and Conn, P.J. (1993). Metabotropic glutamate receptors in brain function and pathology. Trends Pharmacol Sci 14, 13-20.

Schroeder, J.E., Fischbach, P.S., and McCleskey, E.W. (1990). T-type calcium channels: heterogeneous expression in rat sensory neurons and selective modulation by phorbol esters. J Neurosci 10, 947-951.

Schumacher, T.B., Beck, H., Steffens, R., Blumcke, I., Schramm, J., Elger, C.E., and Steinhauser, C. (2000). Modulation of calcium channels by group I and group II metabotropic glutamate receptors in dentate gyrus neurons from patients with temporal lobe epilepsy. Epilepsia 41, 1249-1258.

Shigemoto, R., Abe, T., Nomura, S., Nakanishi, S., and Hirano, T. (1994). Antibodies inactivating mGluR1 metabotropic glutamate receptor block long-term depression in cultured Purkinje cells. Neuron 12, 1245-1255.

Shigemoto, R., Nakanishi, S., and Mizuno, N. (1992). Distribution of the mRNA for a metabotropic glutamate receptor (mGluR1) in the central nervous system: an in situ hybridization study in adult and developing rat. J Comp Neurol 322, 121-135.

Shin, S.L., Hoebeek, F.E., Schonewille, M., De Zeeuw, C.I., Aertsen, A., and De Schutter, E. (2007). Regular patterns in cerebellar Purkinje cell simple spike trains. PLoS ONE 2, e485.

Sidach, S.S., and Mintz, I.M. (2002). Kurtoxin, a gating modifier of neuronal high- and low-threshold ca channels. J Neurosci 22, 2023-2034.

Sillevis Smitt, P., Kinoshita, A., De Leeuw, B., Moll, W., Coesmans, M., Jaarsma, D., Henzen-Logmans, S., Vecht, C., De Zeeuw, C., Sekiyama, N., et al. (2000). Paraneoplastic cerebellar ataxia due to autoantibodies against a glutamate receptor. N Engl J Med 342, 21-27.

Snutch, T.P. (2005). Targeting chronic and neuropathic pain: the N-type calcium channel comes of age. NeuroRx 2, 662-670.

Snutch, T.P., and David, L.S. (2006). T-type calcium channels: an emerging therapeutic target for the treatment of pain. Drug Development Research 67, 404-415.

Snutch, T.P., Leonard, J.P., Gilbert, M.M., Lester, H.A., and Davidson, N. (1990). Rat brain expresses a heterogeneous family of calcium channels. Proc Natl Acad Sci U S A 87, 3391-3395.

Snutch, T.P., Peloquin, J., Mathews, E., and McRory, J.E. (2005). Molecular properties of voltage-gated calcium channels. In Voltage-gated calcium channels, G.W. Zamponi, ed. (New York: Landes Bioscience), pp. 61-94.

Song, I., Kim, D., Choi, S., Sun, M., Kim, Y., and Shin, H.S. (2004). Role of the alpha1G T-type calcium channel in spontaneous absence seizures in mutant mice. J Neurosci 24, 5249-5257.

Soong, T.W., Stea, A., Hodson, C.D., Dubel, S.J., Vincent, S.R., and Snutch, T.P. (1993). Structure and functional expression of a member of the low voltage-activated calcium channel family. Science 260, 1133-1136.

Splawski, I., Yoo, D.S., Stotz, S.C., Cherry, A., Clapham, D.E., and Keating, M.T. (2006). CACNA1H mutations in autism spectrum disorders. J Biol Chem 281, 22085-22091.

Staes, M., Talavera, K., Klugbauer, N., Prenen, J., Lacinova, L., Droogmans, G., Hofmann, F., and Nilius, B. (2001). The amino side of the C-terminus determines fast inactivation of the T-type calcium channel alpha1G. J Physiol 530, 35-45.

Starr, T.V., Prystay, W., and Snutch, T.P. (1991). Primary structure of a calcium channel that is highly expressed in the rat cerebellum. Proc Natl Acad Sci U S A 88, 5621-5625.

Stea, A., Soong, T.W., and Snutch, T.P. (1995). Determinants of PKC-dependent modulation of a family of neuronal calcium channels. Neuron 15, 929-940.

Stea, A., Tomlinson, W.J., Soong, T.W., Bourinet, E., Dubel, S.J., Vincent, S.R., and Snutch, T.P. (1994). Localization and functional properties of a rat brain alpha 1A calcium channel reflect similarities to neuronal Q- and P-type channels. Proc Natl Acad Sci U S A 91, 10576-10580.

Stotz, S.C., Jarvis, S.E., and Zamponi, G.W. (2004). Functional roles of cytoplasmic loops and pore lining transmembrane helices in the voltage-dependent inactivation of HVA calcium channels. J Physiol 554, 263-273.

Sugimori, M., and Llinas, R.R. (1990). Real-time imaging of calcium influx in mammalian cerebellar Purkinje cells in vitro. Proc Natl Acad Sci U S A 87, 5084-5088.

Suh, B.C., and Hille, B. (2005). Regulation of ion channels by phosphatidylinositol 4,5-bisphosphate. Curr Opin Neurobiol 15, 370-378.

Suzuki, S., and Rogawski, M.A. (1989). T-type calcium channels mediate the transition between tonic and phasic firing in thalamic neurons. Proc Natl Acad Sci U S A 86, 7228-7232.

Swartz, K.J., and Bean, B.P. (1992). Inhibition of calcium channels in rat CA3 pyramidal neurons by a metabotropic glutamate receptor. J Neurosci 12, 4358-4371.

Swensen, A.M., and Bean, B.P. (2003). Ionic mechanisms of burst firing in dissociated Purkinje neurons. J Neurosci 23, 9650-9663.

Tai, C., Kuzmiski, J.B., and MacVicar, B.A. (2006). Muscarinic enhancement of R-type calcium currents in hippocampal CA1 pyramidal neurons. J Neurosci 26, 6249-6258.

Takechi, H., Eilers, J., and Konnerth, A. (1998). A new class of synaptic response involving calcium release in dendritic spines. Nature 396, 757-760.

Talavera, K., and Nilius, B. (2006a). Biophysics and structure-function relationship of T-type Ca2+ channels. Cell Calcium 40, 97-114.

Talavera, K., and Nilius, B. (2006b). Evidence for common structural determinants of activation and inactivation in T-type Ca2+ channels. Pflugers Arch 453, 189-201.

Talavera, K., Staes, M., Janssens, A., Droogmans, G., and Nilius, B. (2004). Mechanism of arachidonic acid modulation of the T-type Ca2+ channel alpha1G. J Gen Physiol 124, 225-238.

Talavera, K., Staes, M., Janssens, A., Klugbauer, N., Droogmans, G., Hofmann, F., and Nilius, B. (2001). Aspartate residues of the Glu-Glu-Asp-Asp (EEDD) pore locus control selectivity and permeation of the T-type $\mathrm{Ca}(2+)$ channel alpha(1G). J Biol Chem 276, 45628-45635.

Talley, E.M., Cribbs, L.L., Lee, J.H., Daud, A., Perez-Reyes, E., and Bayliss, D.A. (1999). Differential distribution of three members of a gene family encoding low voltage-activated (T-type) calcium channels. J Neurosci 19, 1895-1911.

Tao, J., Hildebrand, M.E., Liao, P., Liang, M.C., Tan, G., Li, S., Snutch, T.P., and Soong, T.W. (2008). Activation of corticotropin-releasing factor receptor 1 selectively inhibits CaV3.2 T-type calcium channels. Mol Pharmacol.

Tedford, H.W., and Zamponi, G.W. (2006). Direct G protein modulation of Cav2 calcium channels. Pharmacol Rev 58, 837-862.

Tempia, F., Alojado, M.E., Strata, P., and Knopfel, T. (2001). Characterization of the mGluR(1)mediated electrical and calcium signaling in Purkinje cells of mouse cerebellar slices. J Neurophysiol 86, 1389-1397.

Tempia, F., Miniaci, M.C., Anchisi, D., and Strata, P. (1998). Postsynaptic current mediated by metabotropic glutamate receptors in cerebellar Purkinje cells. J Neurophysiol 80, 520-528.

Todorovic, S.M., Jevtovic-Todorovic, V., Mennerick, S., Perez-Reyes, E., and Zorumski, C.F. (2001a). $\mathrm{Ca}(\mathrm{v}) 3.2$ channel is a molecular substrate for inhibition of T-type calcium currents in rat sensory neurons by nitrous oxide. Mol Pharmacol 60, 603-610.

Todorovic, S.M., Jevtovic-Todorovic, V., Meyenburg, A., Mennerick, S., Perez-Reyes, E., Romano, C., Olney, J.W., and Zorumski, C.F. (2001b). Redox modulation of T-type calcium channels in rat peripheral nociceptors. Neuron 31, 75-85.

Tombler, E., Cabanilla, N.J., Carman, P., Permaul, N., Hall, J.J., Richman, R.W., Lee, J., Rodriguez, J., Felsenfeld, D.P., Hennigan, R.F., and Diverse-Pierluissi, M.A. (2006). G protein-induced trafficking of voltage-dependent calcium channels. J Biol Chem 281, 1827-1839.

Toselli, M., and Lux, H.D. (1989). Opposing effects of acetylcholine on the two classes of voltagedependent calcium channels in hippocampal neurons. Exs 57, 97-103.

Toselli, M., and Taglietti, V. (1995). Muscarine inhibits high-threshold calcium currents with two distinct modes in rat embryonic hippocampal neurons. J Physiol 483 (Pt 2), 347-365.

Traboulsie, A., Chemin, J., Chevalier, M., Quignard, J.F., Nargeot, J., and Lory, P. (2007). Subunitspecific modulation of T-type calcium channels by zinc. J Physiol 578, 159-171.

Tsakiridou, E., Bertollini, L., de Curtis, M., Avanzini, G., and Pape, H.C. (1995). Selective increase in Ttype calcium conductance of reticular thalamic neurons in a rat model of absence epilepsy. J Neurosci 15, 3110-3117.

Tseng, G.N., and Boyden, P.A. (1991). Different effects of intracellular Ca and protein kinase C on cardiac T and L Ca currents. Am J Physiol 261, H364-379.

Tsien, R.W., and Barrett, C.F. (2005). A brief history of calcium channel discovery. In Voltage-gated calcium channels, G.W. Zamponi, ed. (New York: Landes Bioscience), pp. 27-47.

Usowicz, M.M., Sugimori, M., Cherksey, B., and Llinas, R. (1992). P-type calcium channels in the somata and dendrites of adult cerebellar Purkinje cells. Neuron 9, 1185-1199.

Vassort, G., Talavera, K., and Alvarez, J.L. (2006). Role of T-type Ca2+ channels in the heart. Cell Calcium 40, 205-220.

Viana, F., Van den Bosch, L., Missiaen, L., Vandenberghe, W., Droogmans, G., Nilius, B., and Robberecht, W. (1997). Mibefradil (Ro 40-5967) blocks multiple types of voltage-gated calcium channels in cultured rat spinal motoneurones. Cell Calcium 22, 299-311.

Vitko, I., Bidaud, I., Arias, J.M., Mezghrani, A., Lory, P., and Perez-Reyes, E. (2007). The I-II loop controls plasma membrane expression and gating of $\mathrm{Ca}(\mathrm{v}) 3.2$ T-type $\mathrm{Ca} 2+$ channels: a paradigm for childhood absence epilepsy mutations. J Neurosci 27, 322-330.

Vitko, I., Chen, Y., Arias, J.M., Shen, Y., Wu, X.R., and Perez-Reyes, E. (2005). Functional characterization and neuronal modeling of the effects of childhood absence epilepsy variants of CACNA1H, a T-type calcium channel. J Neurosci 25, 4844-4855.

Wan, X., Desilets, M., Soboloff, J., Morris, C., and Tsang, B.K. (1996). Muscarinic activation inhibits Ttype Ca2+ current in hen granulosa cells. Endocrinology 137, 2514-2521.

Wang, S.S., Denk, W., and Hausser, M. (2000). Coincidence detection in single dendritic spines mediated by calcium release. Nat Neurosci 3, 1266-1273.

Wang, Y., Rowan, M.J., and Anwyl, R. (1997). LTP induction dependent on activation of Ni2+-sensitive voltage-gated calcium channels, but not NMDA receptors, in the rat dentate gyrus in vitro. J Neurophysiol 78, 2574-2581.

Watanabe, S., Takagi, H., Miyasho, T., Inoue, M., Kirino, Y., Kudo, Y., and Miyakawa, H. (1998). Differential roles of two types of voltage-gated $\mathrm{Ca} 2+$ channels in the dendrites of rat cerebellar Purkinje neurons. Brain Res 791, 43-55.

Weber, J.T., De Zeeuw, C.I., Linden, D.J., and Hansel, C. (2003). Long-term depression of climbing fiber-evoked calcium transients in Purkinje cell dendrites. Proc Natl Acad Sci U S A 100, 2878-2883.

Welker, H.A., Wiltshire, H., and Bullingham, R. (1998). Clinical pharmacokinetics of mibefradil. Clin Pharmacokinet 35, 405-423.

Welsby, P.J., Wang, H., Wolfe, J.T., Colbran, R.J., Johnson, M.L., and Barrett, P.Q. (2003). A mechanism for the direct regulation of T-type calcium channels by Ca2+/calmodulin-dependent kinase II. J Neurosci 23, 10116-10121.

White, G., Lovinger, D.M., and Weight, F.F. (1989). Transient low-threshold Ca2+ current triggers burst firing through an afterdepolarizing potential in an adult mammalian neuron. Proc Natl Acad Sci U S A 86, 6802-6806.

Williams, S.R., Toth, T.I., Turner, J.P., Hughes, S.W., and Crunelli, V. (1997). The 'window' component of the low threshold $\mathrm{Ca} 2+$ current produces input signal amplification and bistability in cat and rat thalamocortical neurones. J Physiol 505 (Pt 3), 689-705.

Wolfart, J., and Roeper, J. (2002). Selective coupling of T-type calcium channels to SK potassium channels prevents intrinsic bursting in dopaminergic midbrain neurons. J Neurosci 22, 3404-3413.

Wolfe, J.T., Wang, H., Howard, J., Garrison, J.C., and Barrett, P.Q. (2003). T-type calcium channel regulation by specific G-protein betagamma subunits. Nature 424, 209-213.

Wolfe, J.T., Wang, H., Perez-Reyes, E., and Barrett, P.Q. (2002). Stimulation of recombinant Ca(v)3.2, T-type, $\mathrm{Ca}(2+$ ) channel currents by CaMKIIgamma(C). J Physiol 538, 343-355.

Womack, M., and Khodakhah, K. (2002). Active contribution of dendrites to the tonic and trimodal patterns of activity in cerebellar Purkinje neurons. J Neurosci 22, 10603-10612.

Womack, M.D., Chevez, C., and Khodakhah, K. (2004). Calcium-activated potassium channels are selectively coupled to P/Q-type calcium channels in cerebellar Purkinje neurons. J Neurosci 24, 88188822.

Womack, M.D., and Khodakhah, K. (2004). Dendritic control of spontaneous bursting in cerebellar Purkinje cells. J Neurosci 24, 3511-3521.

Wu, L., Bauer, C.S., Zhen, X.G., Xie, C., and Yang, J. (2002). Dual regulation of voltage-gated calcium channels by PtdIns(4,5)P2. Nature 419, 947-952.

Xia, J., Chung, H.J., Wihler, C., Huganir, R.L., and Linden, D.J. (2000). Cerebellar long-term depression requires PKC-regulated interactions between GluR2/3 and PDZ domain-containing proteins. Neuron 28, 499-510.

Yao, J., Davies, L.A., Howard, J.D., Adney, S.K., Welsby, P.J., Howell, N., Carey, R.M., Colbran, R.J., and Barrett, P.Q. (2006). Molecular basis for the modulation of native T-type Ca2+ channels in vivo by $\mathrm{Ca} 2+/$ calmodulin-dependent protein kinase II. J Clin Invest 116, 2403-2412.

Yunker, A.M. (2003). Modulation and pharmacology of low voltage-activated ("T-Type") calcium channels. J Bioenerg Biomembr 35, 577-598.

Yunker, A.M., Sharp, A.H., Sundarraj, S., Ranganathan, V., Copeland, T.D., and McEnery, M.W. (2003). Immunological characterization of T-type voltage-dependent calcium channel CaV3.1 (alpha 1G) and CaV3.3 (alpha 1I) isoforms reveal differences in their localization, expression, and neural development. Neuroscience 117, 321-335.

Zamponi, G.W., Bourinet, E., Nelson, D., Nargeot, J., and Snutch, T.P. (1997). Crosstalk between G proteins and protein kinase C mediated by the calcium channel alpha1 subunit. Nature 385, 442-446.

Zamponi, G.W., Bourinet, E., and Snutch, T.P. (1996). Nickel block of a family of neuronal calcium channels: subtype- and subunit-dependent action at multiple sites. J Membr Biol 151, 77-90.

Zhang, Y., Cribbs, L.L., and Satin, J. (2000). Arachidonic acid modulation of alpha1H, a cloned human T-type calcium channel. Am J Physiol Heart Circ Physiol 278, H184-193.

Zhang, Y., Mori, M., Burgess, D.L., and Noebels, J.L. (2002). Mutations in high-voltage-activated calcium channel genes stimulate low-voltage-activated currents in mouse thalamic relay neurons. J Neurosci 22, 6362-6371.

Zhong, X., Liu, J.R., Kyle, J.W., Hanck, D.A., and Agnew, W.S. (2006). A profile of alternative RNA splicing and transcript variation of CACNA1H, a human T-channel gene candidate for idiopathic generalized epilepsies. Hum Mol Genet 15, 1497-1512.

Chapter 2: Inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.3$ by mAChRs

## 2 SELECTIVE INHIBITION OF CAv3.3 T-TYPE CALCIUM CHANNELS BY $G \alpha_{Q / 11}$-COUPLED MUSCARINIC ACETYLCHOLINE RECEPTORS*

### 2.1 Introduction

T-type $\mathrm{Ca}^{2+}$ channels play critical roles in shaping the electrical, chemical and plastic properties of neurons throughout the CNS and PNS. In thalamic nRT and TC neurons, T-type channels are involved in rhythmic rebound burst firing and spindle waves associated with slow-wave sleep (Anderson et al., 2005; Destexhe and Sejnowski, 2003; Huguenard and Prince, 1992; Kim et al., 2001; Tsakiridou et al., 1995). Studies on KO mice and a rat model of absence epilepsy indicate that altering T-type activity within thalamic cells can contribute to pathological conditions such as sleep disorders and epilepsy (Anderson et al., 2005; Destexhe and Sejnowski, 2003; Huguenard and Prince, 1992; Kim et al., 2001; Tsakiridou et al., 1995). Certain human epilepsies appear to be associated with T-type $\mathrm{Ca}^{2+}$ channel point mutations conferring channel gain-of-function phenotypes (Khosravani et al., 2004; Khosravani et al., 2005; Peloquin et al., 2006; Vitko et al., 2005). T-type channels also play crucial roles in dendritic integration and $\mathrm{Ca}^{2+}$ spiking in hippocampal pyramidal cells (Christie et al., 1995; Thompson and Wong, 1991). Within the olfactory bulb, T-type channels are implicated in modulating $\mathrm{Ca}^{2+}$ transients and synaptic release at dendrodendritic synapses (Egger et al., 2003, 2005). In the periphery, antisense oligonucleotides and pharmacological approaches have implicated T-type channels in contributing to both acute and chronic nociceptive behaviors (Bourinet et al., 2005; Hildebrand and Snutch, 2006).

Previous studies have identified three main subtypes of T-type $\mathrm{Ca}^{2+}$ channel $\alpha_{1}$ subunits ( $\mathrm{Ca}_{\mathrm{v}} 3.1$, $\mathrm{Ca}_{\mathrm{v}} 3.2$ and $\mathrm{Ca}_{\mathrm{v}} 3.3$ ) and characterized their voltage-dependent, kinetic, and pharmacological properties (Cribbs et al., 1998; Lee et al., 1999; McRory et al., 2001; Monteil et al., 2000; Perez-Reyes et al., 1998; Santi et al., 2002). Cav 3.1 and $\mathrm{Ca}_{\mathrm{v}} 3.2$ channels display "typical" T-type properties, including relatively small conductance, fast activation and inactivation kinetics and slow deactivation kinetics, while $\mathrm{Ca}_{\mathrm{v}} 3.3$ channels uniquely display a larger conductance, much slower activation and inactivation kinetics as well as faster deactivation kinetics (Lee et al., 1999; McRory et al., 2001). Some of the distinct biophysical properties associated with Cavv.3 T-type currents have been observed in certain populations of native Ttype currents (Huguenard and Prince, 1992; Joksovic et al., 2005; Lee et al., 1999; McRory et al., 2001). The biophysical differences between the T-type channels likely enables them to differentially shape and modulate firing patterns, with the more slowly inactivating $\mathrm{Ca}_{\mathrm{v}} 3.3$ currents able to produce longer bursts of spikes and tonic firing patterns (Chevalier et al., 2006; McRory et al., 2001; Murbartian et al., 2004).

[^0]Although the basic properties of both cloned and native T-type channels have now been largely characterized, there remains relatively little information concerning their modulation by GPCR-linked pathways. Neurotransmitters such as acetylcholine have been shown to either attenuate or stimulate low threshold $\mathrm{Ca}^{2+}$ currents depending on the type of native cells examined, and sometimes multiple forms of modulation can be observed within the same cell type (Castillo et al., 1999; Chemin et al., 2006; Fraser and MacVicar, 1991; Pemberton et al., 2000; Wan et al., 1996). Multiple T-type Ca ${ }^{2+}$ channel subtypes are expressed in most native cells (Talley et al., 1999; Yunker et al., 2003) although pharmacological tools with the specificity needed to separate these currents have not been generated. In this regard, the description of the modulation of specific T-type $\mathrm{Ca}^{2+}$ channels in heterologous systems will provide insights crucial towards further investigations within native systems. This approach is also well-suited for GPCR studies as most neurotransmitters activate multiple receptor subtypes in neurons.

Within thalamic nRT, hippocampal pyramidal and olfactory granule cells, there is evidence for the expression of both T-type $\mathrm{Ca}^{2+}$ channels and $\mathrm{Ga}_{\mathrm{q} / 11}$-coupled mAChRs (Castillo et al., 1999; Levey et al., 1994; Levey et al., 1995; McKay et al., 2006; Plummer et al., 1999; Talley et al., 1999; Wei et al., 1994; Yunker et al., 2003). As both T-type $\mathrm{Ca}^{2+}$ currents and mAChRs have been independently shown to play important physiological roles within these cell types, their functional coupling could be relevant to a number of neuronal processes. In the present paper we studied the modulatory effects of mAChRs on the three main subtypes of low threshold T-type $\mathrm{Ca}^{2+}$ channels expressed in the mammalian nervous system. We find the selective modulation of $\mathrm{Ca}_{\mathrm{v}} 3.3 \mathrm{Ca}^{2+}$ channels by $\mathrm{G}_{\mathrm{q} / 11}$-coupled mAChRs and combined pharmacological, genetic and chimeric channel approaches to examine the G-protein-mediated pathway and structural regions responsible for the distinct $\mathrm{Ca}_{\mathrm{v}} 3.3$ signaling characteristics.

### 2.2 Results

### 2.2.1 Muscarinic M1 receptors selectively inhibit Cave3 T-type calcium channels

To investigate the potential for T-type $\mathrm{Ca}^{2+}$ channel modulation by mAChRs, we transiently transfected HEK cell lines stably expressing individual subtypes of recombinant rat brain T-type channels with the human muscarinic M1 receptor. Perforated patch recordings with $\beta$-Escin demonstrated that activation of M1 with 1 mM carbachol (CCh) caused a rapid ( $<30 \mathrm{sec}$ ) and robust inhibition of exogenously expressed rat brain Cav 3.3 T-type channel peak currents ( $-45 \%+/-2 \%, n=34$ ) (Fig. 2.1C,F). Only a small subpopulation of stable $\mathrm{Ca}_{\mathrm{v}} 3.3$ cells ( $<10 \%$ ) were not affected by CCh application (likely representing cells untransfected with the M1 receptor). Activation of M1 with 1 mM CCh had no significant effect ( $\mathrm{p}>0.05$ ) on the voltage-dependence of Ca 3.3 currents, but significantly increased both the rates of activation and inactivation ( $\mathrm{p}<0.001$; Table 2.1). In contrast to the clear inhibition of Cav 3.3 T-type currents, activation of M1 receptors with 1 mM CCh largely had no effect on
the peak current amplitude of either rat brain $\mathrm{Ca}_{\mathrm{v}} 3.1(-2.1 \%+/-2.0 \%, \mathrm{n}=18)$ or $\mathrm{Ca}_{\mathrm{v}} 3.2$ channels $(-0.1 \%$ $+/-2.3 \%, \mathrm{n}=17$ ) (Fig. 2.1A,B,D,E).

In a small subset of both $\mathrm{Ca}_{\mathrm{v}} 3.1$ and $\mathrm{Ca}_{\mathrm{v}} 3.2$ currents we noted a stimulation induced by M1 activation $\left(\mathrm{Ca}_{\mathrm{v}} 3.1=35 \%+/-12 \%, \mathrm{n}=4 ; \mathrm{Ca}_{\mathrm{v}} 3.2=36 \%+/-12 \%, \mathrm{n}=5\right)$, with a slower time course to equilibrium of greater than one minute ( $n=3$ and $n=4$, respectively). For the prevalent null effect on $\mathrm{Ca}_{\mathrm{v}} 3.1$ and $\mathrm{Ca}_{\mathrm{v}} 3.2$ currents, 1 mM CCh application had no significant effect on channel activation and inactivation kinetics or the voltage-dependence of activation ( $\mathrm{p}>0.05$; Table 2.1).

Different Cav 3.3 T-type channel isoforms with distinct carboxyl termini have been identified from both the rat and human brain (Lee et al., 1999; McRory et al., 2001; Monteil et al., 2000; Murbartian et al., 2002). To test whether inhibition of the Cav 3.3 channel by M1 receptors was restricted to the rat brain short carboxyl terminus isoform (McRory et al., 2001), we also examined the longer human $\mathrm{Ca}_{\mathrm{v}} 3.3$ isoform (Monteil et al., 2000) transiently co-transfected into HEK cells with the M1 receptor. Similar to that for the shorter rat brain isoform, application of 1 mM CCh resulted in significant inhibition of the human $\mathrm{Ca}_{\mathbf{v}} 3.3$ peak current amplitude ( $-28 \%+/-2 \%, \mathrm{n}=15$ ) and also significantly increased activation and inactivation kinetics (p<0.001; Fig. 2.5A; Table 2.1). Additionally, similar to that for the rat $\mathrm{Ca}_{\mathrm{v}} 3.1$ T-type channel, application of 1 mM CCh to HEK cells co-transfected with the human $\mathrm{Ca}_{\mathrm{v}} 3.1$ channel and M1 receptor had no significant effect on peak current amplitude ( $0.3 \%+/-2.0 \%, \mathrm{n}=9$ ) or channel kinetics (for $100 \%$ cells tested; $\mathrm{p}>0.05$; Fig. 2.7B; Table 2.1). Overall, the differential modulation of T-type $\mathrm{Ca}^{2+}$ channel subtypes mediated by M1 receptors was consistent across both rat and human recombinant T-type channels.

Table 2.1 - Effects of Receptor Activation on T-Type Channel Kinetic and VoltageDependent Properties

|  | 2mM Ca ${ }^{\text {2+ }}$ Control |  |  | 1 mM Carbachol |  |  |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: |
|  | $\tau_{\text {act }}(\mathrm{ms})$ | $\tau_{\text {inact }}(\mathrm{ms})$ | $\mathrm{V}_{50 \text { act }}(\mathrm{mV})$ | $\tau_{\text {act }}(\mathrm{ms})$ | $\tau_{\text {inact }}(\mathrm{ms})$ | $\mathrm{V}_{50 \text { act }}(\mathrm{mV})$ |
| Cav3.3 + M1 ${ }_{\text {(Inhibition) }}$ | $6.0+/-0.4, n=27$ | $86+/-6, n=27$ | $-51+/-2, n=13$ | $4.1+/-0.3, \mathrm{n}=27^{* *}$ | $31+/-2, n=27^{*}$ | $-49+/-1, n=13$ |
| human Cav3.3 + M1 (Whole-Cell; Inhibition) | $9.7+/-0.8, n=10$ | $117+/-6, n=10$ | $-44+/-1, n=4$ | $5.4+/-0.6, n=10^{* *}$ | $41+/-5, n=10^{* *}$ | -47 +/-1, n=4 |
| Cav3.3 + M2 (No Effect) | $6.1+/-0.7, n=10$ | $81+/-9, n=10$ | -53+/-3, n=4 | $5.5+/-0.7, n=10$ | $76+/-9, n=10$ | $-52+/-3, n=4$ |
| Cav3.3 + M3 (Inhibition) | $9.5+/-0.9, n=9$ | $104+/-13, n=9$ | -53 +/-4, n=4 | $6.2+/-0.6, n=9$ * | $56+/-6, n=8$ * | $-50+/-4, n=4$ |
| Cav3.3 + M4 (No Effect) | $6.7+/-0.9, n=6$ | $110+/-18, n=6$ | -50 +/-1, n=5 | $6.0+/-0.9, n=6$ | $101+/-16, n=6$ | $-51+/-2, n=5$ |
| Cav3.3 + M5 (Inhibition) | $8.6+/-0.9, n=6$ | $127+/-23, n=7$ | $-48+/-1, n=5$ | $5.6+/-0.6, n=6$ | $49+/-6, n=7 *$ | $-45+/-1, n=5$ |
| Cav3.3 + Control Plasmid | $6.0+/-0.4, n=5$ | $78+/-13, n=5$ |  | $5.2+/-0.2, n=5$ | $71+/-12, n=5$ |  |
| Cav3.1-M1 ${ }_{\text {(No Effect) }}$ | $2.3+/-0.2, n=17$ | $19+/-2, n=17$ | -36 +/-2, n=17 | $2.1+/-0.2, n=17$ | $17+/-1, \mathrm{n}=17$ | -39 +/-3, n=11 |
| human Cav3.1 + M1 ${ }_{\text {(Whole-Cell; }}$ No Effect) | $2.0+/-0.4, n=9$ | $14+/-1, n=9$ | -42+/-2, n=6 | $1.2+/-0.1, n=9$ | $13+/-1, n=9$ | $-51+/-3, n=4$ |
| Cav3.2 + M1 ${ }_{\text {(No Effect) }}$ | $4.7+/-0.2, n=16$ | $36+/-2, n=15$ | $-42+/-1, n=7$ | $4.3+/-0.2, n=16$ | $34+/-2, n=15$ | $-40+/-2, n=7$ |

* $\mathrm{p}<0.02$, ** $\mathrm{p}<0.001$


Figure 2.1 - T-type calcium channels are differentially modulated by M1 receptors.
$\mathbf{A}, \mathbf{B})$ Representative perforated patch current traces during depolarizing pulses from -110 mV to -30 mV demonstrating no effect on $\mathrm{Ca}_{\mathrm{v}} 3.1$ currents $(\mathbf{A})$ and $\mathrm{Ca}_{\mathrm{v}} 3.2$ currents $(\mathbf{B})$ when M 1 is activated with 1 mM CCh. D,E) Normalized peak current levels during perfusion of control recording solution ( $2 \mathrm{mM} \mathrm{Ca}^{2+}$ ) followed by 1 mM CCh for $\mathrm{Ca}_{\mathrm{v}} 3.1(+\mathrm{M} 1)$ currents (D) and Cav $3.2(+\mathrm{M} 1)$ currents (E). Perfusion of CCh usually had no effect on $\mathrm{Ca}_{\mathrm{v}} 3.1$ peak current amplitudes ( $-2.1 \%+/-2.0 \%, \mathrm{n}=18$ ) and $\mathrm{Ca}_{\mathrm{v}} 3.2$ peak current amplitudes ( $-0.1 \%+/-2.3 \%, \mathrm{n}=17$ ). C) Representative perforated patch current traces during depolarizing pulses from -110 mV to -40 mV showing inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.3$ currents by M1.
F) Normalized peak current levels during perfusion of control recording solution $\left(2 \mathrm{mM} \mathrm{Ca}{ }^{2+}\right)$ followed by 1 mM CCh for $\mathrm{Ca}_{\mathrm{v}} 3.3$ (+M1) currents. Perfusion of CCh caused a $45 \%(+/-2 \%, \mathrm{n}=34)$ decrease in

Ca 3.3 currents. All data points correspond to mean $+/-$ S.E.

### 2.2.2 Muscarinic M1 receptors dose-dependently modulate $\mathrm{Ca}_{\mathbf{v}} 3.3$ biophysical properties

Perforated patch recordings on stable rat $\mathrm{Ca}_{\mathrm{v}} 3.3$ cells transiently transfected with M1 receptors revealed that the CCh -induced inhibition of peak current levels was reversible over a time course of about 2 minutes ( $\mathrm{n}=13$; Fig. 2.2A,B). As previously mentioned, activation of M1 receptors with 1 mM CCh caused a significant ( $\mathrm{p}<0.001$ ) increase in inactivation kinetics (Control, $\tau_{\text {inact }}=86+/-6, \mathrm{n}=27 ; 1 \mathrm{mM}$ $\mathrm{CCh}, \tau_{\text {inact }}=31+/-2, \mathrm{n}=27$ ). Along with peak current inhibition, the CCh-induced increase in Cave 3.3 inactivation kinetics was reversible (Fig. 2.2A,C). Both the M1 receptor-induced inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.3$ peak currents and the increased inactivation rate would be predicted to reduce the total amount of $\mathrm{Ca}^{2+}$ flowing through $\mathrm{Ca}_{\mathrm{v}} 3.3$ T-type channels during a cellular depolarization. The effect of M1 receptor activation on total $\mathrm{Ca}^{2+}$ influx was determined by integrating the area over $\mathrm{Ca}_{\mathrm{v}} 3.3$ current traces during 200 ms depolarizing pulses to peak potential before and after 1 mM CCh application. Normalizing these $\mathrm{Ca}^{2+}$ influx values to control levels showed a $77 \%+/-2 \%(n=20)$ reduction in $\mathrm{Ca}^{2+}$ influx mediated by M1 receptor activation (Fig. 2.2D).

As activation of M1 receptors increases the kinetics of $\mathrm{Ca}_{\mathrm{v}} 3.3$ activation and inactivation, it is possible that this signaling pathway modulates $\mathrm{Ca}_{\mathrm{v}} 3.3$ function via acting upon the open and/or inactivated states. A protocol that involved perfusion of 1 mM CCh for 50 seconds (time to reach normal inhibition equilibrium) in the absence of test pulses, followed by regular 0.2 Hz test pulses to peak potential examined whether the M1 receptor-mediated inhibitory effect is use-dependent. Figure 2.2E shows that this "no depolarization" protocol displayed the same level of inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.3$ currents observed in the regular 0.2 Hz experiments, indicating that M1 effects on $\mathrm{Ca}_{\mathrm{v}} 3.3$ are useindependent. Increasing the test pulse frequency to 0.5 Hz also caused no significant ( $\mathrm{p}>0.05$ ) change in the level of $\mathrm{Ca}_{\mathrm{v}} 3.3$ inhibition, indicating that the M 1 effects on $\mathrm{Ca}_{\mathrm{v}} 3.3$ are also frequency-independent (Fig. 2.2E). Another possibility is that M1 receptor activation inhibits $\mathrm{Ca}_{\mathrm{v}} 3.3$ currents by shifting steadystate inactivation to more hyperpolarized potentials, reducing the proportion of channels available (in the closed state) to open at the holding potential of -110 mV . A protocol with a 1 second prepulse to -140 mV to remove accumulated channel inactivation demonstrated no significant ( $\mathrm{p}>0.05$ ) difference in inhibition compared to the control protocol, suggesting that the inhibitory effect is not due to changes in the steady-state inactivation of $\mathrm{Ca}_{\mathrm{v}} 3.3$ channels (Fig. 2.2E).

Control experiments with mock transfections of an empty control vector or with a pre-incubated mAChR antagonist (atropine) demonstrated that the CCh-induced inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.3$ currents is mediated specifically via the transfected M1 receptor (Fig. 2.2E). Testing the effects of varying concentrations of CCh on stable $\mathrm{Ca}_{\mathrm{v}} 3.3$ cells with transfected M1 receptors revealed that the inhibitory effect is dose-dependent (Fig. 2.2F). The $\mathrm{IC}_{50}$ for inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.3$ currents by $\mathrm{CCh}=27 \mu \mathrm{M}$, consistent with that reported for phophatidyl inositol hydrolysis triggered by M1 receptor activation in both HEK 293 and CHO cells (Hogger et al., 1995; Schwarz et al., 1993).


Figure 2.2 - Mechanistic properties of inhibition of $\mathrm{Ca}_{\mathbf{v}} \mathbf{3} 3$ currents by M1 receptors.
A) The inhibition of $\mathrm{Ca}_{v} 3.3$ channels by M1 is reversible. Representative $\mathrm{Ca}_{\mathrm{v}} 3.3$ perforated patch current traces during depolarizing pulses from -110 mV to -40 mV before (1), during (2), and after (3) perfusion of 1 mM CCh. Note the increase in inactivation kinetics when CCh is applied (Table 2.1). B) A plot of peak current amplitude (for same cell as in A) showing the rate of inhibition by 1 mM CCh perfusion and the rate of washout, with the selected traces from $\mathbf{A}(1,2,3)$ labeled. C) Application of 1 mM CCh increases $\mathrm{Ca}_{\mathrm{v}} 3.3$ inactivation kinetics in a reversible manner. The inactivating component of every trace from $\mathbf{B}$ was fit with an exponential equation to give $\tau_{\text {inact }}$. D) Application of 1 mM CCh dramatically reduces the amount of $\mathrm{Ca}^{2+}$ influx through $\mathrm{Ca}_{v} 3.3$ channels. The effects of M1 activation on normalized $\mathrm{Ca}^{2+}$ influx is shown for all $\mathrm{Ca}_{\mathrm{v}} 3.3$ cells. Perfusion of 1 mM CCh caused a $77 \%(+/-2 \%$, $\mathrm{n}=20$ ) decrease in $\mathrm{Ca}^{2+}$ influx. E) Inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.3$ currents by 1 mM CCh occurs through M1 receptors. Control experiments show elimination of the inhibition due to 1 mM CCh when a muscarinic antagonist ( $50 \mu \mathrm{M}$ atropine) is co-applied, or when a control vector (pBluescript) is transfected instead of M1. A lack of depolarizing test pulses during initial CCh perfusion, increase in test pulse frequency to 0.5 Hz , or a hyperpolarizing prepulse to -140 mV for 1 second had no significant ( $\gg 0.02$ ) effect on the magnitude of $\mathrm{Ca}_{\mathrm{v}} 3.3$ inhibition by M1. F) CCh inhibited $\mathrm{Ca}_{\mathrm{v}} 3.3$ currents in a dose-dependent manner. CCh concentration vs. percentage block data was fit with a Hill equation and the $\mathrm{IC}_{50}$ for CCh inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.3$ currents was $27 \mu \mathrm{M}$. All data points correspond to mean $+/-$ S.E.

### 2.2.3 Inhibition of $\mathbf{C a} \mathbf{v} \mathbf{3} 3$ channels by M1 receptors requires $\mathbf{G} \alpha_{q / 11}$

The inhibitory effect of M1 receptor activation on $\mathrm{Ca}_{\mathrm{v}} 3.3$ currents could occur either through $\mathrm{G} \beta \gamma$ - or $\mathrm{G} \alpha$-mediated processes. To test for the involvement of $\mathrm{G} \beta \gamma$, a membrane-targeted version of the C-terminus of $\beta$ ARK, MAS-GRK3ct (Kammermeier et al., 2000), was co-transfected with M1 receptors into the stable $\mathrm{Ca}_{\mathrm{v}} 3.3$ HEK cell line. Control experiments showed that the MAS-GRK3ct construct was able to completely abolish the well described $\mathrm{G} \beta \gamma$-dependent inhibition of N -type $\mathrm{Ca}^{2+}$ channels (data not shown). MAS-GRK3ct only partially reduced M1-mediated Cav 3.3 current inhibition ( $-24.8 \%+/-$ $3.4 \%, \mathrm{n}=10$ ) in most cells, suggesting that inhibition is distinct from the previously reported pure $\mathrm{G} \beta \gamma$ mediated inhibition of N - and $\mathrm{P} / \mathrm{Q}-$ type and $\mathrm{Ca}_{\mathrm{v}} 3.2 \mathrm{Ca}^{2+}$ channels (Dolphin, 2003; Wolfe et al., 2003) (Fig. 2.3A,D). A smaller subset of MAS-GRK3ct co-transfected cells displayed no exponential inhibitory effect $(-9.3 \%+/-3.0 \%, \mathrm{n}=7)$. Co-expression of transducin $\left(G \alpha_{\mathrm{t}}\right)$, which also buffers $\mathrm{G} \beta \gamma$ signaling (Kammermeier et al., 2000), caused the same reduction in M1-mediated inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.3$ currents $(-25.1 \%+/-2.5 \%, \mathrm{n}=10)$, with a small number of cells not being inhibited at all $(7.0 \%+/-$ $10.1 \%$, n=3) (Fig. 2.3B,D).

In contrast to the partial effect of G $\beta \gamma$ signaling antagonists, co-expression of the Regulator of G-Protein Signaling 2, RGS2 - an effector antagonist for $G \alpha_{q / 11}$ (Heximer et al., 1997), completely prevented the M1 receptor-induced inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.3$ currents for all cells examined. In perforated patch recordings of $\mathrm{Ca}_{\mathrm{v}} 3.3$ cells co-transfected with M1 and RGS2, application of 1 mM CCh either had no effect (Fig. 2.3C,D; $1 \%+/-5 \%, n=7$ ), or caused a stimulation of Cav 3.3 currents ( $30 \%+/-9 \%, n=5$ ).

### 2.2.4 Constitutively active $\mathbf{G} \alpha_{q / 11}$ proteins modulate Cav 3.3 T-type calcium channels

To further test whether active $\mathrm{G}_{\mathrm{q} / 11} \mathrm{G}$-proteins are sufficient to produce inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.3$ currents, stable $\mathrm{Ca}_{\mathrm{v}} 3.3$ cells were transiently transfected with various constitutively active $\mathrm{G} \alpha$ subunit constructs. These constructs contain missense mutations that confer constitutive activity by reducing GTPase activity. If $\alpha_{q / 11}$ is the downstream signal of M 1 receptor activation mediating the effects on $\mathrm{Ca}_{\mathrm{v}} 3.3$ currents then it is hypothesized that activation of the co-expressed $\mathrm{G}_{\mathrm{q}}$ or $\mathrm{G}_{11}$ mutants by dialysis of GTP would cause a reduction in current amplitude and an increase in inactivation kinetics. Similar to a study that analyzed inhibition of KCNQ2/KCNQ3 channels by $\mathrm{G}_{\mathrm{q} / 11}$ (Suh et al., 2004), we used constitutively active $\mathrm{G} \alpha_{\mathrm{q}}\left(\mathrm{G} \alpha_{q-\mathrm{Q} 209 \mathrm{~L}}\right)$ and $\mathrm{G} \alpha_{11}\left(\mathrm{G} \alpha_{11-\mathrm{Q} 209 \mathrm{~L}}\right)$ mutants to test for the hypothesized effect and a constitutively active $\mathrm{G} \alpha$ protein $\left(\mathrm{G} \alpha_{13-\mathrm{Q} 226 \mathrm{~L}}\right)$ that does not couple to the same downstream effectors (PLC) as a negative control. We also performed controls wherein empty vectors were transfected.


$$
\mathrm{B} \quad \mathrm{Cav} 3.3+\mathrm{M} 1+\mathrm{G} \alpha_{\mathrm{t}}
$$



D


Figure 2.3-Inhibition of $\mathrm{Ca}_{\mathbf{v}} \mathbf{3 . 3}$ channels by M1 requires $\mathbf{G} \alpha_{q / 11}$ signaling.
A) In perforated patch recordings of $\mathrm{Ca}_{\mathbf{v}} 3.3$ stable cells co-transfected with M1 and a membrane targeted form of the C-terminus of $\beta$ ARK that sequesters active G $\beta \gamma$ subunits (MAS-GRK3ct), application of 1 mM CCh caused inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.3$ currents $(-24.8 \%+/-3.4 \%, \mathrm{n}=10)$. B) Similarly, inhibition by perfusion of 1 mM CCh was observed for $\mathrm{Ca}_{\mathrm{v}} 3.3$ cells co-transfected with the $\mathrm{G} \beta \gamma$ buffer $\mathrm{G} \alpha_{\text {transducin }}$ $\left(G \alpha_{\mathrm{t}}\right)$ and M1 $(-25.1 \%+/-2.5 \%, \mathrm{n}=10)$. C) In perforated patch recordings of Ca 3.3 cells co-transfected with M1 and RGS2 (antagonist of active G $\alpha_{q / 11}$ subunits), application of 1 mM CCh predominantly had no effect $(1 \%+/-5 \%, n=7)$ on $\mathrm{Ca}_{\mathrm{v}} 3.3$ currents. D) Bar graph comparing various genetic and pharmacological manipulations to control conditions where stable $\mathrm{Ca}_{\mathrm{v}} 3.3$ cells are transfected with M1 and inhibited by 1 mM CCh. Inhibitors of serine/threonine kinases ( 500 nM staurosporine, $\mathrm{n}=7 ; 50 \mu \mathrm{M}$ H9, $n=6$ ), PKC ( $10 \mu$ M Chelerythrine, $n=5 ; 500 \mathrm{nM}$ Go 6976, $\mathrm{n}=6$ ), tyrosine kinases ( $10 \mu \mathrm{M}$ genistein, $\mathrm{n}=7$ ), phosphatases ( 100 nM okadaic acid, $\mathrm{n}=6$ ), phosphoinositide-3-kinases ( 200 nM wortmannin, $\mathrm{n}=7$ ), PTX-sensitive G $\alpha$ proteins ( $0.5 \mu \mathrm{~g} / \mathrm{mL}$ PTX, $\mathrm{n}=6$ ), cAMP ( $10 \mu \mathrm{M}$ Rp-cAMPs, $\mathrm{n}=5$ ) and internal $\mathrm{Ca}^{2+}$ $\left(10 \mu\right.$ M BAPTA-AM, $n=5$ ) had no significant ( $\mathrm{p}>0.02$ ) effect on the inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.3$ currents by M1. RGS2, MAS-GRK3ct, and $\mathrm{G} \alpha_{\mathrm{t}}(\mathrm{p}<0.001)$ caused a significant elimination or reduction in the inhibition of $\mathrm{Ca}_{v} 3.3$ currents by M1. All data points correspond to mean $+/-$ S.E.

By comparing traces 30 seconds after forming the whole-cell configuration with traces 2 minutes after whole-cell in Figure 2.4A-D, we found that dialysis of the cell with the GTP-containing pipette internal solution caused both a significant reduction in peak current levels and an increase in inactivation kinetics only for the $\mathrm{G} \alpha_{\text {q-Q209L }}$ and $\mathrm{G} \alpha_{11-\mathrm{Q} 209 \mathrm{~L}}$ transfections. The ratio of peak current levels at 2 minutes divided by the peak current levels at 30 seconds was significantly reduced ( $\mathrm{p}<0.001$ ) for $\mathrm{G} \alpha_{\mathrm{q} \text {-Q209L }}(\mathrm{n}=16)$ and $\operatorname{Ga}_{11-\text {-209L }}(\mathrm{n}=15)$ compared to the control transfection $(\mathrm{n}=18)$, while the $\mathrm{G} \alpha_{13-\mathrm{Q} 226 \mathrm{~L}}(\mathrm{n}=17)$ transfection current ratio was not significantly altered ( $(\mathrm{p}>0.05)$, Fig. 2.4E)). The rates of inactivation ( $\tau_{\text {inact }}$ ) were determined during depolarizing steps from -110 mV to -30 mV for all transfection types. The $\tau_{\text {unact }}$ was significantly faster ( $\mathrm{p}<0.001$ ) for $\mathrm{G} \alpha_{\mathrm{q}-\mathrm{Q} 209 \mathrm{~L}}$ and $\mathrm{G} \alpha_{11-\mathrm{Q} 209 \mathrm{~L}}$ compared to control transfections, while the $\tau_{\text {tract }}$ was not significantly different for $\mathrm{G} \alpha_{13-\mathrm{Q} 226 \mathrm{~L}}$ ( $\mathrm{p}>0.02$; Fig. 2.4F).

### 2.2.5 $\mathrm{Ga}_{q^{\prime} / 11}$ inhibits $\mathrm{Ca}_{\mathbf{v}} \mathbf{3 . 3}$ channels through an unidentified non-classical pathway

The active GTP-bound form of $\mathrm{G} \alpha_{q / 11}$ causes the activation of PLC, which then produces $\mathrm{IP}_{3^{-}}$ and DAG/PKC-mediated signals. Various pharmacological antagonists were used to investigate the role of potential cellular signals downstream of $G \alpha_{q / 11}$. Specific inhibitors of PKC including $10 \mu \mathrm{M}$ chelerythrine ( $\mathrm{n}=5$ ) and 500 nM Go $6976(\mathrm{n}=6)$ had no significant ( $\mathrm{p}>0.05$ ) effect on the M1-mediated inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.3$ currents (Fig. 2.3D). To ensure pharmacological activity of these antagonists, the PKC-mediated stimulation of Cav 3.2 channels by 300 nM PMA ( $65 \%+/-17 \%$, $\mathrm{n}=7$; see (Park et al., 2006) ) was shown to be significantly ( $\mathrm{p}<0.02$ ) abolished by both $10 \mu \mathrm{M}$ chelerythrine $(-15 \%+/-6 \%$, $\mathrm{n}=5)$ and 500 nM Go $6976(3 \%+/-5 \%, \mathrm{n}=5)$ (data not shown). Inhibitors of serine/threonine kinases ( 500 nM staurosporine, $n=7 ; 50 \mu \mathrm{MH9}, \mathrm{n}=6$ ), tyrosine kinases ( $10 \mu \mathrm{M}$ genistein, $n=7$ ), phosphatases ( 100 nM okadaic acid, $\mathrm{n}=6$ ), phosphoinositide-3-kinases ( 200 nM wortmannin, $\mathrm{n}=7$ ), PTX-sensitive $\mathrm{G} \alpha$ proteins $(0.5 \mu \mathrm{~g} / \mathrm{mL}$ PTX, $\mathrm{n}=6)$, cAMP ( $10 \mu \mathrm{M}$ Rp-cAMPs, $\mathrm{n}=5$ ) and internal $\mathrm{Ca}^{2+}(10 \mu \mathrm{M}$ BAPTA-AM, $\mathrm{n}=5$ ) also had no significant ( $\mathrm{p}>0.02$ ) effect on the inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.3$ currents by M1 receptors (Fig. 2.3D). In this regard, classical $\mathrm{Ga}_{q / 11}$ downstream effectors such as PKC, PKA, and increased cytosolic $\mathrm{Ca}^{2+}$ concentration appear not to be directly involved in the M1 receptor-mediated inhibition of Cav 3.3 Ttype $\mathrm{Ca}^{2+}$ currents. Phospholipase C activity has recently been shown to directly inhibit voltage-gated ion channels through the depletion of membrane $\mathrm{PIP}_{2}$ levels, which are thought to stabilize active channels in the membrane (Suh and Hille, 2005). Dialyzing cells with a $\operatorname{PIP}_{2}$ antibody ( $50 \mu \mathrm{~g} / \mathrm{mL}$ ) to reduce available $\mathrm{PIP}_{2}$ levels had no significant effect ( $\mathrm{p}>0.05$ ) on the M1-receptor mediated inhibition of Cav 3.3 currents (Fig. 2.5B,D). Similarly, dialyzing cells with synthetic PIP $_{2}(200 \mu \mathrm{M}$ di-C8 PIP 2 ) to saturate membrane $\mathrm{PIP}_{2}$ levels also had no significant effect ( $\mathrm{p}>0.05$ ) on M1 receptor-mediated inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.3$ currents (Fig. 2.5C,D).


Figure 2.4 - The $\mathbf{G} \alpha_{q / 11}$ subtypes of $\mathbf{G} \alpha$ proteins specifically cause inhibition of $\mathrm{Ca}_{\mathbf{v}} \mathbf{3 . 3}$ currents. A-D) Representative whole-cell current traces of stable $\mathrm{Ca}_{\mathrm{v}} 3.3$ cells transfected with various control or $\mathrm{G} \alpha$ plasmids during depolarizing steps from -110 mV to -30 mV . Traces were obtained 30 seconds (black) and 2 minutes (grey) after the whole-cell conformation was formed, using an internal solution that contained 4 mM ATP and 0.3 mM GTP. The stable $\mathrm{Ca}_{\mathrm{v}} 3.3$ cells were mock-transfected with empty plasmid (A) or transfected with the constitutively active forms (lack of GTPase activity) of G $\alpha$-proteins:
$\left.\mathrm{G} \alpha_{13-\mathrm{Q} 226 \mathrm{~L}}(\mathbf{B}), \mathrm{G} \alpha_{q-\mathrm{Q} 209 \mathrm{~L}}(\mathbf{C}), \mathrm{G} \alpha_{11-\mathrm{Q} 209 \mathrm{~L}}(\mathbf{D}) . \mathbf{E}\right) \mathrm{G} \alpha_{q-\mathrm{Q} 209 \mathrm{~L}}$ and $\mathrm{G} \alpha_{11-\mathrm{Q} 209 \mathrm{~L}}$ cause a time-dependent reduction in $\mathrm{Ca}_{\mathrm{v}} 3.3$ current magnitude. The peak current levels at 2 minutes were divided by the peak current levels at 30 seconds to determine the level of inhibition due to internal solution dialysis for the various types of transfected $\mathrm{Ca}_{\mathrm{v}} 3.3$ cells, as described above. The $\mathrm{Ca}_{\mathrm{v}} 3.3$ currents co-transfected with $\mathrm{G} \alpha_{q-\mathrm{Q} 209 \mathrm{~L}}$ and $\mathrm{G} \alpha_{11-\mathrm{Q} 209 \mathrm{~L}}$ had a significant ( $\mathrm{p}<0.001$ ) reduction in current ratio compared to the control transfection, while the $\mathrm{G} \alpha_{13-\text {-Q26L }}$ transfection caused no significant ( $\mathrm{p}>0.05$ ) change. F) The rate of inactivation ( $\tau_{\text {inatt }}$ ) was determined during depolarizing steps from -110 mV to -30 mV for all transfection types. The $\tau_{\text {mact }}$ was significantly ( $\mathrm{p}<0.001$ ) faster for $\mathrm{G} \alpha_{\mathrm{q}-\mathrm{Q} 209 \mathrm{~L}}$ and $\mathrm{G} \alpha_{11-\mathrm{Q} 209 \mathrm{~L}}$ compared to control transfections, while the $\tau_{\text {mact }}$ was not significantly ( $\mathrm{p}>0.02$ ) different for $\mathrm{G} \alpha_{13 \text {-Q226L }}$. All data points correspond to mean $+/$ S.E.


Figure 2.5 - Inhibition of $\mathbf{C a}_{\mathbf{v}} \mathbf{3 . 3}$ channels by M1 does not require $\mathrm{PIP}_{\mathbf{2}}$ signaling. A) In whole-cell recordings of HEK 293 cells co-transfected with human $\mathrm{Ca}_{\mathrm{v}} 3.3$ and M1, application of 1 mM CCh caused inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.3$ currents ( $-27.7 \%+/-1.5 \%, \mathrm{n}=15$ ). B) Dialyzing cells with 50 $\mu \mathrm{g} / \mathrm{mL} \mathrm{PIP}_{2}$ antibody for $10+$ minutes had no effect on the inhibition of human $\mathrm{Ca}_{\mathrm{v}} 3.3$ currents by M1 receptor activation $(-30.8 \%+/-3.9 \%, \mathrm{n}=7)$. C) Dialyzing cells with $200 \mu \mathrm{M}$ di-C8 PIP 2 for $5+$ minutes had no effect on the inhibition of human $\mathrm{Ca}_{\mathrm{v}} 3.3$ currents by M1 receptor activation ( $-29.0 \%+/-1.8 \%$, $\mathrm{n}=6$ ). D) Bar graph showing that attenuating $\mathrm{PIP}_{2}$ signaling with either $\mathrm{PIP}_{2}$ antibodies or di-C8 $\mathrm{PIP}_{2}$ had no significant effect ( $\mathrm{p}>0.05$ ) on M1-mediated inhibition compared to whole-cell recordings from control human $\mathrm{Ca}_{\mathrm{v}} 3.3+\mathrm{M} 1$ cells.

As a positive control for di-C8 PIP $_{2}$ activity and as previously shown (Bian et al., 2001), dialysis of $200 \mu \mathrm{M}$ di-C8 PIP ${ }_{2}$ into HEK 293 cells stably expressing HERG $\mathrm{K}^{+}$channels caused a significant ( $\mathrm{p}<0.02$ ) stimulation of $\mathrm{K}^{+}$channel currents $(\mathrm{n}=8)$ compared to control recordings ( $\mathrm{n}=5$; data not shown). Taken together, these results indicate that inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.3$ by M1 receptors occurs either directly through $\mathrm{G} \alpha_{q / 11}$, or a downstream pathway that is independent of $\mathrm{PIP}_{2}$ metabolism and other classical effectors.

### 2.2.6 G $\alpha_{q / 11}$-coupled muscarinic receptors selectively inhibit $\mathrm{Ca}_{\mathbf{v}} \mathbf{3 . 3} \mathbf{~ c h a n n e l s}$

If inhibition of Ca 3.3 T -type $\mathrm{Ca}^{2+}$ channels by M 1 receptors is primarily dependent on $\mathrm{G} \alpha_{q / 11}$ signaling, then all $\mathrm{G} \alpha_{q} / \mathrm{G}_{11}$-coupled mAChRs should similarly inhibit $\mathrm{Ca}_{\mathrm{v}} 3.3$ currents, while $\mathrm{Ga}_{\mathrm{i}}-$ coupled mAChRs should have no effect. Indeed, activation of co-expressed $\mathrm{Ga}_{\mathrm{i}}$-coupled M2 and M4 receptors with 1 mM CCh had no effect on $\mathrm{Ca}_{\mathrm{v}} 3.3$ current amplitude ( $\mathrm{M} 2=-4 \%+/-2 \%, \mathrm{n}=11 ; \mathrm{M} 4=-$ $4 \%+/-3 \%, n=8$ ) or kinetics (Fig. 2.6A,C,E,G; Table 2.1). In contrast, upon transfection of either the $\mathrm{G} \alpha_{\mathrm{q}} / \mathrm{G} \alpha_{11}$-coupled M3 or M5 receptor subtypes into stable Ca 3.3 cells perforated patch recordings revealed a significant CCh-mediated inhibition ( $\mathrm{M} 3=-25 \%+/-3 \%, \mathrm{n}=10$; $\mathrm{M} 5=-31 \%+/-3 \%, \mathrm{n}=10$ ) as well as a concomitant increase in both activation and inactivation kinetics (Fig. 2.6B,D,F,H; Table 2.1). Overall, experiments with genetically encoded antagonists of $\mathrm{G}_{\mathrm{q} / 11}$ (RGS2) and G $\beta \gamma$ (MASGRK3ct) and genetically-encoded G $\alpha$ subtypes, as well as inhibition experiments with various mAChRs all support the assertion that inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.3$ channels by mAChRs specifically occurs through $\mathrm{G} \alpha_{q / 11}$.

### 2.2.7 Two distinct $\mathrm{Ca}_{\mathrm{v}} 3.3$ channel regions are involved in M1-mediated inhibition

Most modulation of $\mathrm{Ca}^{2+}$ channels by intracellular signaling pathways involves physical interactions between various effectors and cytoplasmic channel domains (Wolfe et al., 2003; Zamponi and Snutch, 2002). Chimeric T-type $\mathrm{Ca}^{2+}$ channels between human $\mathrm{Ca}_{\mathrm{v}} 3.1$ and human $\mathrm{Ca}_{\mathrm{v}} 3.3$ were generated to determine the molecular regions of the $\mathrm{Ca}_{\mathrm{v}} 3.3$ channel involved in the M1 receptormediated inhibition (Fig. 2.7). The $\mathrm{Ca}_{\mathrm{v}} 3.1$ and $\mathrm{Ca}_{\mathrm{v}} 3.3$ full-length channels were initially divided into 4 approximately equal portions and chimeric channels were constructed using restriction enzyme digestion and re-ligation (see (Hamid et al., 2006)). The four channel portions were named as followed: Region 1 $=$ amino-terminus + domain I, Region $2=$ domain I-II linker, domain II + the first 39-63 amino acids of the domain II-III linker, Region $3=$ remainder of the domain II-III linker + domain III, and Region $4=$ the domain III-IV linker, domain IV + the carboxyl-terminus. Chimeric channel names were assigned based on whether the chimera contained $\mathrm{Ca}_{\mathrm{v}} 3.1$ (G) or $\mathrm{Ca}_{\mathrm{v}} 3.3$ (I) sequence in each of the four regions described (e.g., the chimeric $\mathrm{Ca}_{\mathrm{v}} 3.3$ channel that contained Region 2 from $\mathrm{Ca}_{\mathrm{v}} 3.1$ is called IGII).


Figure 2.6 - Inhibition of $\mathrm{Ca}_{\mathbf{v}} \mathbf{3} 3$ currents occurs specifically through $\mathbf{G} \alpha_{q / 11}$-coupled mAChRs. A,C) Representative perforated patch current traces during depolarizing pulses from -110 mV to -40 mV showing no effect on $\mathrm{Ca}_{\mathrm{v}} 3.3$ currents by the $\mathrm{Ga}_{\mathrm{i}}$-coupled M2 and M4 receptors, respectively. Traces during control perfusion and perfusion of 1 mM CCh are indistinguishable and CCh application had no significant effect on channel kinetics (Table 2.1). E,G) Averaged time course of normalized peak current levels during perfusion of control recording solution $\left(2 \mathrm{mM} \mathrm{Ca}^{2+}\right)$ followed by 1 mM CCh for $\mathrm{Ca}_{\mathrm{v}} 3.3(+\mathrm{M} 2 / \mathrm{M} 4)$ currents. Perfusion of CCh had no effect on $\mathrm{Ca}_{\mathrm{v}} 3.3$ currents for M2 ( $\mathbf{E} ;-4 \%+/-2 \%$, $\mathrm{n}=11)$ and $\mathrm{M} 4(\mathbf{G} ;-4 \%+/-3 \%, \mathrm{n}=8)$ receptors. B,D) Representative perforated patch current traces during depolarizing pulses from -110 mV to -40 mV showing inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.3$ currents by the $\mathrm{G} \alpha_{q / 11}$-coupled M3 and M5 receptors, respectively. Activating the receptors with 1 mM CCh also significantly ( $\mathrm{p}<0.02$ ) increased channel kinetics (Table 2.1). F,H) Averaged time course of normalized peak current levels during perfusion of control recording solution $\left(2 \mathrm{mM} \mathrm{Ca}^{2+}\right)$ followed by 1 mM CCh for $\mathrm{Ca}_{\mathrm{v}} 3.3$ plus either M3 or M5 receptors. Perfusion of CCh caused a $25 \%+/-3 \%$ decrease ( $\mathrm{n}=10$ ) of $\mathrm{Ca}_{\mathrm{v}} 3.3$ currents with co-transfected M3 receptors ( $\mathbf{F}$ ), and a $31 \%+/-3 \%$ decrease ( $\mathrm{n}=10$ ) of $\mathrm{Ca}_{\mathrm{v}} 3.3$ currents with co-transfected M5 receptors. Only a small number of cells ( $\mathrm{n}=2$ for both M3 and M5) were not inhibited by CCh. All data points correspond to mean $+/$ S.E.


## Figure 2.7-Regions II and IV of human $\mathrm{Ca}_{\mathbf{v}} \mathbf{3} 3$ channels are required and appear sufficient for M1-mediated inhibition.

A-D) (Left) Schematic diagrams of the various chimeric channels that were co-transfected into HEK cells with M1. The blue transmembrane domains and intra/extra-cellular regions correspond to Cav 3.3 (labeled I) sequences, while the red transmembrane domains and intra/extra-cellular regions correspond to $\mathrm{Ca}_{\mathrm{v}} 3.1$ (labeled G) sequences. (Middle) The effect of activating M1 receptors with 1 mM CCh on the normalized peak current levels of chimeric channel types shown to the left. Inclusion of $\mathrm{Ca}_{\mathrm{v}} 3.1$ sequence at Regions II and IV (C) eliminated M1-mediated inhibition and attenuated the effect on inactivation kinetics, while inclusion of Cav 3.3 sequence at Regions II and IV (D) restored M1-mediated inhibition to a level that was not significantly ( $\mathrm{p}>0.05$ ) different from IIII inhibition levels (see Table 2.1). (Right)
Insets include chimeric whole-cell current traces during depolarizing pulses from -110 mV to peak potential before (line arrow) and after (block arrow) application of 1 mM CCh . Traces are representative of the various chimeras in terms of activation and inactivation kinetics as well as magnitude of inhibition. For inset scale bars, $x=50 \mathrm{~ms}, \mathrm{y}=100 \mathrm{pA}$. E) Histogram where GIII, IGII, IIGI, IIIG, and IGIG inhibition values were statistically compared to the IIII control while IGGG, GIGG, GGIG, and GGGI values were compared to the GGGG control and the GIGI value was compared to both the IIII and GGGG controls. * indicates significant difference ( $<0.02$ ) compared to IIII inhibition, while ** indicates a significant difference ( $\mathrm{p}<0.02$ ) compared to GGGG modulation levels. All data points correspond to mean $+/-$ S.E.

Co-expression of M1 receptors with chimeric GIII and IIGI T-type channels both showed a similar degree of M1 receptor-mediated peak current inhibition compared to that of the inhibition of the wt Cavv 3.3 channel (IIII) (Fig. 2.7A,E). In contrast, when the IGII chimera was co-transfected with M1 receptors, application of 1 mM CCh resulted in a significantly attenuated degree of inhibition $(-5.6 \%+/-$ $2.1 \%, \mathrm{n}=11,(\mathrm{p}<0.001))$ compared to the wt IIII channel ( $-26.9 \%+/-2.3 \%, \mathrm{n}=9$; Fig. 2.7E).
Interestingly, while the chimeric IGII channels exhibited lowered M1 receptor-mediated inhibition they still possessed significantly increased inactivation kinetics (p<0.001; Table 2.2). Finally, while the IIIG chimeric channels showed similar degree of M1 receptor-mediated peak current inhibition compared to the wt IIII, the rate of inhibition was notably slower (not shown). The changes in the rate of inhibition for IIIG and the significant decrease in the amount of inhibition for IGII suggested that both Regions 2 and 4 might be involved in the M1-induced inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.3$ channels. To explore this, a double chimera (IGIG) was co-transfected into HEK cells with M1 receptors. Figures 2.7C and 2.7E show that the inhibiting effect of 1 mM CCh application on peak current amplitude was completely abolished for the IGIG chimera $(0.9 \%+/-2.5 \%, \mathrm{n}=8)$. Activation of M1 with 1 mM CCh still caused a significant increase ( $\mathrm{p}<0.001$ ) in the inactivation kinetics of IGIG, but the $\tau_{\text {inact }}$ decreased by less than $25 \%$ for IGIG while it decreased by $40-65 \%$ for all the single chimeric and wt $\mathrm{Ca}_{\mathrm{v}} 3.3$ channels (Table 2.2).

The chimeric channel loss-of-function experiments indicate that both Regions 2 and 4 are involved in the M1-mediated inhibition of current amplitude and increase in inactivation kinetics of $\mathrm{Ca}_{\mathrm{v}} 3.3$ currents. In gain-of-function experiments, substitution of either Region 2 (GIGG) or Region 4 (GGGI) into the $\mathrm{Ca}_{\mathrm{v}} 3.1$ channel resulted in 1 mM CCh -induced inhibition (GIGG $=-14.3 \%+/-0.8 \%$, $\mathrm{n}=7$; GGGI $=-9.1 \%+/-2.6 \%, \mathrm{n}=9$ ) that was significantly different ( $\mathrm{p}<0.001$ and $\mathrm{p}<0.02$, respectively) when compared to GGGG ( $-0.3 \%+/-2.2 \%$, $\mathrm{n}=9$; Fig. 2.7B,E; Table 2.2). In contrast, inclusion of either Region 1 or Region 3 of $\mathrm{Ca}_{\mathrm{v}} 3.3$ into $\mathrm{Ca}_{\mathrm{v}} 3.1$ resulted in no significant change ( $\mathrm{p}>0.05$ ) in M1-mediated inhibition when compared to GGGG (Fig. 2.7E). Although both GIGG and GGGI were inhibited by M1, the level of inhibition was significantly lower ( $\mathrm{p}<0.001$ ) than the inhibition of IIII by M1 (Fig. 2.7E). When the effect of 1 mM CCh application on GIGI current amplitude was tested, M1 activation was found to produce a significant level of GIGI inhibition $(-25.1 \%+/-2.4 \%, \mathrm{n}=11 ; \mathrm{p}<0.001)$ compared to GGGG that was not significantly ( $\mathrm{p}>0.05$ ) different from the inhibition of IIII (Fig. 2.7A,B,D,E). Application of 1 mM CCh also significantly increased ( $\mathrm{p}<0.001$ ) the rate of inactivation for the GIGI $\mathrm{Ca}_{\mathrm{v}} 3.1$ chimera, but not for GIGG, GGGI, or the other Cav 3.1 single chimeras (Table 2.2). Overall, the combined substitution of Regions 2 and 4 from the $\mathrm{Ca}_{\mathrm{v}} 3.3$ channel into the $\mathrm{Ca}_{\mathrm{v}} 3.1$ channel completely restores M1-induced inhibition together with the associated increase in channel inactivation kinetics.

## Table 2.2-Effects of M1 Receptor Activation on Chimeric T-Type Channel Inactivation Kinetics



* $\mathrm{p}<0.02$, ${ }^{* *} \mathrm{p}<0.001$


### 2.3 Discussion

In the present paper we systematically explored the effects of activated mAChRs on the three main T-type $\mathrm{Ca}^{2+}$ channel isoforms expressed in the mammalian nervous system and report for the first time the differential modulation between a G-protein signaling pathway and Cav 3.3 T-type $\mathrm{Ca}^{2+}$ channels. Most studies on T-type $\mathrm{Ca}^{2+}$ channel modulation have involved the $\mathrm{Ca}_{\mathrm{v}} 3.2\left(\alpha_{1 \mathrm{H}}\right)$ subtype, revealing specific modulatory responses to $\mathrm{G} \beta_{2} \gamma$, CAMKII, and redox modulation that are not observed for the $\mathrm{Ca}_{\mathrm{v}} 3.1$ and $\mathrm{Ca}_{\mathrm{v}} 3.3$ T-type $\mathrm{Ca}^{2+}$ channel isoforms (Joksovic et al., 2006; Welsby et al., 2003; Wolfe et al., 2003). The exclusive inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.3$ channels by $\mathrm{G} \alpha_{q / 11}$-coupled mAChRs is the first example of specific, GPCR-mediated modulation of a T-type $\mathrm{Ca}^{2+}$ channel subtype other than for $\mathrm{Ca}_{\mathrm{v}} 3.2$.

### 2.3.1 Differential effects of mAChRs on T-type calcium channel isoforms

Examination of the literature shows that activation of mAChRs can result in multiple effects on native T-type $\mathrm{Ca}^{2+}$ currents including causing stimulation (Fisher and Johnston, 1990; Fraser and MacVicar, 1991; Pemberton et al., 2000), inhibition (Wan et al., 1996), or having no effect (Allen and Brown, 1993). Given the heterogeneous nature of native low threshold $\mathrm{Ca}^{2+}$ currents, without investigating interactions between specific mAChR gene products and specific T-type $\mathrm{Ca}^{2+}$ channel isoforms, the published differences in modulation are nearly impossible to interpret. Our results using exogenous expression of cloned T-type $\mathrm{Ca}^{2+}$ channels indicates that M 1 receptor activation has a robust inhibitory effect on $\mathrm{Ca}_{\mathrm{v}} 3.3$ currents and has either no effect or a small stimulation on both $\mathrm{Ca}_{\mathrm{v}} 3.1$ and $\mathrm{Ca}_{\mathrm{v}} 3.2$ currents. Similarly, experiments examining native $\mathrm{Ca}_{v} 3.2 \mathrm{Ca}^{2+}$ channels in NIH3T3 cells transiently transfected with mAChRs demonstrated that M1 receptor activation had either no effect or a stimulatory effect if a PKC inhibitor was applied (Pemberton et al., 2000). Active G $\beta_{2} \gamma$ subunits have been shown to specifically inhibit Cav 3.2 currents (DePuy et al., 2006; Wolfe et al., 2003) and the lack of inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.2$ channels by M1 receptors in our study is likely due to the absence of any functional coupling between M1 receptors and $\mathrm{G} \beta_{2}$ proteins (Dippel et al., 1996). We also found that all $\mathrm{G} \alpha_{\mathrm{q} / 1^{-}}$ coupled mAChR subtypes (M1, M3, and M5) cause attenuation of $\mathrm{Ca} \sqrt{\mathrm{v}} 3.3$ currents while $\mathrm{Ga}_{\mathrm{i}}$-coupled

M2 and M4 receptors had no effect on $\mathrm{Ca}_{\mathrm{v}} 3.3$ currents. Thus it is likely that any stimulation of T-type $\mathrm{Ca}^{2+}$ currents by mAChRs in native systems does not involve $\mathrm{Ca}_{\mathrm{v}} 3.3$ channels. Experiments testing the effects of recombinant M2-M5 receptors on the $\mathrm{Ca}_{\mathrm{v}} 3.2$ and $\mathrm{Ca}_{\mathrm{v}} 3.1 \mathrm{Ca}^{2+}$ channel isoforms in a heterologous system are required to further facilitate the possibility of interactions between these T-type channels and mAChRs.

### 2.3.2 Functional effects of M1 receptor activation on $\mathrm{Ca}_{\mathbf{v}} \mathbf{3} \mathbf{3} \mathbf{3}$ currents

Activation of M1 receptors dramatically altered $\mathrm{Ca}_{\mathrm{v}} 3.3$ currents by both reversibly attenuating peak current levels and increasing the rate of inactivation, resulting in a significant reduction in the influx of $\mathrm{Ca}^{2+}$. The relationship between these effects was explored using both structural channel chimeras and classical gating property studies. In chimeric studies (see section 2.3.4), the activation of M1 receptors primarily caused an increase in inactivation kinetics of the IGII chimera and conversely, primarily a decrease in peak current levels for the GIGG chimera. Both this isolation of the two specific M1 receptor-mediated effects and the gating results discussed below suggest that the effects of M1 on current amplitude and inactivation kinetics are complementary but distinct phenomena. For gating studies, reduction of $\mathrm{Ca}_{\mathrm{v}} 3.3$ current magnitude by M1 receptor activation was equally robust when the $\mathrm{Ca}_{\mathrm{v}} 3.3$ channels were held in various states: (1) during a prolonged hyperpolarization with no test depolarizations (channels mostly in closed state), (2) after a strong hyperpolarizing prepulse to -140 mV , and (3) during 200 ms test depolarizations to peak potential at 0.2 Hz and 0.5 Hz . Combining this lack of use-dependence with the observed reduction in peak current amplitude and the increase in activation and inactivation kinetics indicates that all states of the $\mathrm{Ca}_{\mathrm{v}} 3.3$ channel are subject to modulation by M1 receptor activation. The acceleration of $\mathrm{Ca}_{\mathrm{v}} 3.3$ channel kinetics by M1 receptor activation also supports the hypothesis that modulation affects channel biophysical properties and not channel density via internalization, which has recently been shown to occur for the voltage-independent, GPCR-mediated inhibition of N -type $\mathrm{Ca}^{2+}$ channels on a relatively fast timescale (Tombler et al., 2006). Physiologically, the combined decrease in $\mathrm{Ca}_{\mathrm{v}} 3.3$ peak currents and the increased activation and inactivation kinetics would be predicted to alter neuronal firing patterns and perhaps eliminate rhythmic oscillations (Chemin et al., 2002; Chevalier et al., 2006). In support of this notion, the concomitant reduction in peak current and increase in inactivation kinetics of $\mathrm{Ca}_{\mathrm{v}} 3.3$ currents triggered by anandamide has been shown to completely eliminate the sustained, rhythmic $\mathrm{Ca}_{\mathrm{v}} 3.3$ current during an AP voltage clamp experiment with an oscillating thalamic waveform (Chemin et al., 2001).

### 2.3.3 Signal transduction pathway of M1 receptor-mediated Cav3.3 inhibition

Use of genetically-encoded antagonists of G $\beta \gamma$ (MAS-GRK3ct and $\mathrm{G}_{\mathrm{t}}$ ) and $\mathrm{G} \alpha_{\mathrm{q} / 11}$ (RGS2) demonstrated that G $\beta \gamma$ may partially contribute to the M1-mediated inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.3$ currents, while $\mathrm{G} \alpha_{q / 11}$ is absolutely required for complete inhibition. The potential involvement of both $\mathrm{G} \alpha_{q / 11}$ and $\mathrm{G} \beta \gamma$ in a non-classical, voltage-independent mechanism of $\mathrm{Ca}^{2+}$ channel inhibition by mAChR has been previously described for $\mathrm{HVA} \mathrm{Ca}^{2+}$ channels. In rat SCG sympathetic neurons application of a muscarinic agonist causes the voltage-independent inhibition of endogenous N -type $\mathrm{Ca}^{2+}$ channels that is abolished by co-expression of RGS2, G $\alpha_{t}$, or MAS-GRK3ct and exhibits a time course similar to the $\mathrm{Ca}_{\mathrm{v}} 3.3$ inhibition reported here (Kammermeier et al., 2000). As $\mathrm{G} \beta \gamma$ is a cofactor for PLC $\beta$ activity, a possible explanation is that sequestering G $\beta \gamma$ reduces PLC $\beta$ activity (Rhee and Bae, 1997). Although $\mathrm{G} \beta \gamma$ may potentiate the inhibitory effect of M1 receptor activation, transfection of constitutively active $\mathrm{G} \alpha_{q / 11}$ mutants into stable $\mathrm{Ca} \mathrm{v}_{\mathrm{v}} 3.3$ cells demonstrated that active $\mathrm{G} \alpha_{q / 11}$ alone is sufficient to induce the inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.3$ currents. In support of this notion, only $\mathrm{G}_{\mathrm{q} / 11}$-coupled mAChRs (M1, M3, and M5) inhibited $\mathrm{Ca}_{\mathrm{v}} 3.3$ currents, while $\mathrm{Ga}_{\mathrm{i}}$-coupled M 2 and M 4 receptors that activate $\mathrm{G} \beta \gamma$ signaling have no effect on $\mathrm{Ca}_{\mathrm{v}} 3.3$ currents. Unlike that reported for the attenuation of $\mathrm{Ca}_{\mathrm{v}} 3.2$ channels by $\mathrm{G}_{2} \gamma$, this novel form of T-type $\mathrm{Ca}^{2+}$ channel inhibition involves the $\mathrm{G} \alpha_{q / 11}$ subunit and also affects channel kinetics. This inhibitory mechanism for the Cav 3.3 T-type isoform may be applicable to all $\mathrm{G} \alpha_{q / 11}$-coupled receptors as we have also found a similar inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.3$ channels by mGluR1a receptors (Hildebrand et al., 2005) (Fig. 3.1).

Pharmacological antagonists eliminated the potential involvement of various intracellular signals downstream of $\mathrm{G} \alpha_{q / 11}$ activation that may be involved in the inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.3$ by M1 receptor activation. Abolishing the activity of PKC, serine/threonine kinases (including PKA), tyrosine kinases, phosphatases, phosphoinositide-3-kinases and intracellular $\mathrm{Ca}^{2+}$ signaling all had no effect on inhibition. This profile of $\mathrm{M} 1 / \mathrm{G} \alpha_{q / 11}$-mediated $\mathrm{Ca}^{2+}$ channel inhibition resistant to common antagonists of cytoplasmic signaling is not unique and has been reported for the inhibition of L-type channels by $\mathrm{G}_{\mathrm{q}^{-}}{ }^{-}$ coupled M1/3/5 receptors in HEK cells (Bannister et al., 2002). Like the inhibition of Ca 3.3 via M1 receptors, this inhibition is voltage-independent, relatively slow kinetically ( $\tau_{\text {on }}=13$ seconds), and insensitive to antagonists of protein kinases and protein phosphatases (Bannister et al., 2002).

A more recent explanation for the $\mathrm{G} \alpha_{q / 11}$-mediated inhibition of ion channels including voltagegated $\mathrm{K}^{+}$channels and HVA Ca ${ }^{2+}$ channels has emerged wherein channel activity is suppressed through the depletion of membrane $\mathrm{PIP}_{2}$ levels via PLC activity (Gamper et al., 2004; Suh et al., 2004; Wu et al., 2002). In these studies, $\mathrm{G} \alpha_{q / 11}$-mediated inhibition was shown to be inhibited via dialysis of synthetic $\mathrm{PIP}_{2}$ or a $\mathrm{PIP}_{2}$-specific antibody into the cytoplasm. In our experiments, adding di-C8 $\mathrm{PIP}_{2}$ or the $\mathrm{PIP}_{2}$ antibody into the internal pipette solution and dialyzing cells for up to 25 minutes had no significant
effect on M1-mediated inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.3$ channels, suggestive of another to-be-defined mechanism whereby $\mathrm{G} \alpha_{q / 11}$ signaling causes the inhibition of voltage-gated ion channels. Further biophysical and biochemical experiments are required to clarify the nature of the intracellular messengers and/or scaffolding proteins that can modulate $\mathrm{Ca}_{v} 3.3 \mathrm{~T}$-type $\mathrm{Ca}^{2+}$ channels and also whether $\mathrm{G}_{\mathrm{q} / 11}$ can interact directly with the channel through a novel mechanism.

### 2.3.4 $\mathbf{G} \alpha_{q / 11}$-mediated inhibition of $\mathrm{Ca}_{\mathbf{v}} \mathbf{3 . 3}$ involves two discrete channel regions

Replacing both Regions 2 and 4 in the $\mathrm{Ca}_{\mathrm{v}} 3.3$ channel with the corresponding Cav 3.1 T -type $\mathrm{Ca}^{2+}$ channel sequences abrogated both the M1 receptor-mediated peak current inhibition and concomitant increase in inactivation kinetics. Conversely, substituting Regions 2 and 4 from $\mathrm{Ca}_{\mathrm{v}} 3.3$ into $\mathrm{Ca}_{\mathrm{v}} 3.1$ conferred M1 receptor-mediated inhibition and increased inactivation kinetics. These data suggest that Regions 2 and 4 of the $\mathrm{Ca}_{\mathrm{v}} 3.3$ channel are both necessary and sufficient to recapitulate M1 receptormediated channel modulation. Region 2 of the $\mathrm{Ca}_{\mathrm{v}} 3.1$ and $\mathrm{Ca}_{\mathrm{v}} 3.3$ sequence contains the highly divergent domain I-II linker, the highly conserved domain II and 39-63 amino acids of the domain II-III linker while Region 4 contains most of the III-IV linker, the highly conserved domain IV and the highly divergent C-terminus. Based on their putatively intracellular regions and their high divergence between the two T-type isoforms, the I-II linker, proximal region of the II-III linker, the III-IV linker, and the Cterminus are all candidates for modulation sites within Regions 2 and 4. Interestingly, the only identified sites of alternative splicing within the rat and human $\mathrm{Ca}_{\mathrm{v}} 3.3$ channel occur both in the I-II linker and the C-terminal regions (Mittman et al., 1999; Murbartian et al., 2002) (Fig. 1.4). The effects of these splicing variations on the biophysical properties (activation kinetics) of the human $\mathrm{Ca}_{\mathrm{v}} 3.3$ channel are interdependent rather than additive, suggesting a possible direct interaction between the I-II linker and Cterminus that affects channel kinetics (Murbartian et al., 2004). Both the human and rat $\mathrm{Ca}_{\mathrm{v}} 3.3$ channels inhibited by M1 receptor activation in our study lack exon 9 located in the I-II linker, while both the rat and human $\mathrm{Ca}_{\mathrm{v}} 3.1$ channels have a 10 amino acid insertion in this region in a manner similar to that for the + exon $9 \mathrm{Ca}_{\mathrm{v}} 3.3$ splice variant. Thus, several observations suggest that the I-II linker may be a target region in the inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.3$ by M1 and some evidence points to a possible role for the C -terminus. However, as multiple structural determinants contribute to the slow inactivation kinetics of $\mathrm{Ca}_{\mathrm{v}} 3.3$ compared to $\mathrm{Ca}_{\mathrm{v}} 3.1$ in a nearly additive manner (Hamid et al., 2006), and M1 activation dramatically speeds up $\mathrm{Ca}_{\mathrm{v}} 3.3$ inactivation kinetics, it is also possible that multiple intracellular loci within Regions 2 and 4 of the $\mathrm{Ca}_{\mathrm{v}} 3.3$ channel may be involved in the M1-mediated effect.

In summary, we find that activation of known $\mathrm{G} \alpha_{q / 11}$-coupled mAChRs results in the selective inhibition of Ca v.3 T-type $\mathrm{Ca}^{2+}$ currents with a concomitant increase in inactivation kinetics. The $\mathrm{G}_{\mathrm{q} / 11}$-mediated signaling pathway appears to be mediated via two disparate regions of the $\mathrm{Ca}_{\mathrm{v}} 3.3$ channel. Functional interactions between mAChRs and $\mathrm{Ca}_{\mathrm{v}} 3.3 \mathrm{Ca}^{2+}$ channels could potentially impact
firing patterns of various cell types including thalamic nRT cells. Both biophysical and pharmacological evidence suggests that primarily $\mathrm{Ca}_{\mathrm{v}} 3.3$ channels compose dendritic T-type currents in nRT cells (Joksovic et al., 2005) while immunostaining suggests the presence of M3 receptors in these cells (Levey et al., 1994). This raises the possibility that the inhibition of Cav 3.3 T-type $\mathrm{Ca}^{2+}$ channels by M3 receptors in the dendrites of nRT cells could be involved in cholinergic modulation of thalamic firing patterns.

### 2.4 Experimental procedures

### 2.4.1 Molecular biology

Human $\mathrm{Ca}_{\mathrm{v}} 3.1-\mathrm{Ca}_{\mathrm{v}} 3.3$ T-type $\mathrm{Ca}^{2+}$ channel $\alpha_{1}$ subunit chimeras were constructed as described in detail by Hamid et al (Hamid et al., 2006).

### 2.4.2 Cell culture and transfection

HEK 293H (Invitrogen, ON, Canada) cells were grown in standard DMEM ( $10 \%$ fetal bovine serum and $50 \mathrm{U} / \mathrm{ml}$ penicillin-streptomycin) to $\sim 80 \%$ confluence and maintained at $37^{\circ} \mathrm{C}$ in a humidified incubator with $95 \%$ atmosphere and $5 \% \mathrm{CO}_{2}$. The generation of stable T-type cell lines (in HEK 293, tsa-201) expressing rat brain $\mathrm{Ca}_{\mathrm{v}} 3.1, \mathrm{Ca}_{\mathrm{v}} 3.2$, or $\mathrm{Ca}_{\mathrm{v}} 3.3 \alpha_{1}$ subunits has been previously described (Santi et al., 2002). Stable cell lines were transiently transfected with human muscarinic M1, M2, M3, M4, or M5 cDNAs (all in pcDNA3.1) using Lipofectamine (Invitrogen). As a reporter for transfection, all transient transfections included co-transfection with either CD8 or pEGFP marker plasmids at a 1:4 molar ratio compared to receptor and/or channel plasmid DNA, unless otherwise indicated. Lipofectamine-mediated transfections used 1 to $1.25 \mu \mathrm{~g}$ of DNA/35 mm dish and $5 \mu \mathrm{~L}$ of Lipofectamine/dish. In G-protein experiments, stable Cav 3.3 cells were co-transfected with M1 receptors and equal amounts of either MAS-GRK3ct (in pcDNA3.1), $\mathrm{Ga}_{\mathrm{t}}$ (in pcDNA3.1) or RGS2 (in pEGFP) using Lipofectamine. Only MAS-GRK3ct and $\mathrm{G}_{\mathrm{t}}$ transfections required co-transfection with marker plasmids as RGS2 expression could be directly detected with fluorescence. In G $\alpha$ transfection experiments, stable $\mathrm{Ca}_{\mathrm{v}} 3.3$ cells were transfected with constitutively active mutants of $\mathrm{G} \alpha_{q}, \mathrm{G} \alpha_{11}$ or $\mathrm{G} \alpha_{13}$ ( $\mathrm{G} \alpha_{q-\mathrm{Q} 209 \mathrm{~L}}, \mathrm{G} \alpha_{11-\mathrm{Q} 209 \mathrm{~L}}$, and $\mathrm{G} \alpha_{13-\mathrm{-Q226L}}$, respectively, all in pcDNA3.1) using Lipofectamine. Twelve to 18 hours after transfection the media was changed from Opti-Mem I to regular DMEM and cells were transferred to a $28^{\circ} \mathrm{C}$ incubator. The M1 to $\mathrm{M} 5, \mathrm{G} \alpha_{\text {q-Q209L }}, \mathrm{G} \alpha_{11-\mathrm{Q} 209 \mathrm{~L}}$, and $\mathrm{G} \alpha_{13-\mathrm{Q} 226 \mathrm{~L}} \mathrm{cDNAs}$ were all obtained from the UMR cDNA Resource Center (Rolla, MO) while the RGS2 and MAS-GRK3ct constructs were a generous gift from Dr. Brett Adams.

In separate experiments, HEK 293H cells were co-transfected with M1 and wt or chimeric human $\mathrm{Ca}_{v} 3.1$ or $\mathrm{Ca}_{v} 3.3$ channels using standard $\mathrm{Ca}^{2+}$ phosphate transfection with $2 \mu \mathrm{~g}$ of total
cDNA/dish, 0.15 to $0.4 \mu \mathrm{~g}$ channel cDNA/dish, and $0.2 \mu \mathrm{~g}$ M1 cDNA/dish. In a subset of these experiments involving co-transfection of wt $\mathrm{Ca}_{\mathrm{v}} 3.3$ and M 1 , either $200 \mu \mathrm{M}$ di- $\mathrm{C} 8 \mathrm{PI}(4,5) \mathrm{P}_{2}$ (Echelon Biosciences Inc., Salt Lake City, UT) or $50 \mu \mathrm{~g} / \mathrm{mL} \operatorname{PI}(4,5) \mathrm{P}_{2} \mathrm{IgG}_{2 \mathrm{~b}}$ antibody ( $\sim 1: 30$ dilution) (Assay Designs, Ann Arbor, MI) was included in the internal solution to explore the role of $\mathrm{PIP}_{2}$ signaling. As the $\mathrm{PIP}_{2}$ antibody was supplied in a PBS buffer solution containing $10 \%$ calf serum and $0.05 \%$ sodium azide, the control $\mathrm{Ca}_{\mathrm{v}} 3.3+\mathrm{M} 1$ cells were recorded in an internal solution containing a 1:30 dilution of PBS with $10 \%$ fetal bovine serum and $0.05 \%$ sodium azide. Electrophysiological recordings for all experiments were performed 24-48 hours after transfection. Transiently transfected cells were selected for CD8 or pEGFP expression using either adherence of Dynabeads (Dynal, Great Neck, NY) or fluorescence of EGFP under UV light.

### 2.4.3 Electrophysiological recordings and analysis

Macroscopic currents were recorded using the perforated patch-clamp technique to reduce current rundown and to preserve cytoplasmic signaling pathways. The external recording solution contained (in mM): $2 \mathrm{CaCl}_{2}, 1 \mathrm{MgCl}_{2}$, 10 HEPES, $40 \mathrm{TEACl}, 92 \mathrm{CsCl}, 10$ glucose, $\mathrm{pH}=7.4$, while the internal pipette solution contained (in mM): 120 CsMethanesulfonate, 11 EGTA, 10 HEPES, $2 \mathrm{MgCl}_{2}$, $75-100 \mu \mathrm{M} \beta$-Escin $\mathrm{pH}=7.2$. For these perforated patch recordings, experimental recording did not begin until the $\mathrm{R}_{\mathrm{S}}$ was below $20 \mathrm{M} \Omega$ and constant, as measured by amplifier compensation. Whole-cell recordings were used for the transiently transfected wt or chimeric human $\mathrm{Ca}_{\mathrm{v}} 3.1$ and $\mathrm{Ca}_{\mathrm{v}} 3.3$. channel experiments as well as the $\mathrm{G} \alpha$ transfection experiments. The internal solution for these recordings contained (in mM): 120 CsMethanesulfonate, 11 EGTA, 10 HEPES, $2 \mathrm{MgCl}_{2}, 4 \mathrm{Mg}$-ATP, 0.3 Na -GTP. Macroscopic currents were recorded using Axopatch 200A and 200B amplifiers (Axon Instruments, Foster City, CA), controlled and monitored with Pentium 4 personal computers running pClamp software version 9 (Axon Instruments). Patch pipettes (borosilicate glass BF150-86-10; Sutter Instruments, Novato, CA), were pulled using a Sutter P-87 puller and polished with a Narishige (Tokyo, Japan) microforge, with typical resistances of 3-6 M $\Omega$ when filled with internal solution. The bath was connected to the ground via a 3 M KCl agar bridge.

Data were low-pass filtered at 2 kHz using the built-in Bessel filter of the amplifier, with sampling at 10 kHz . The amplifier was also used for capacitance and series resistance compensation between $70 \%$ and $85 \%$ on every cell. Leak subtraction of capacitance and leakage current was performed on-line using a $\mathrm{P} / 5$ protocol, or else performed with Clampfit (Axon Instruments) during offline analysis. Figures and fittings utilized the software program Microcal Origin (Version 7.5, Northampton, MA). All recordings were performed at room temperature $\left(20-22^{\circ} \mathrm{C}\right)$.

The voltage-dependence of activation for $\mathrm{Ca}_{\mathrm{v}} 3.1, \mathrm{Ca}_{\mathrm{v}} 3.2$, and $\mathrm{Ca}_{\mathrm{v}} 3.3$ currents were measured by a series of 100 ms to 220 ms depolarizing pulses applied from a holding potential of -110 mV to membrane potentials from -80 mV to +10 mV , increasing at 5 mV increments, with 2 seconds between pulses. The potential that elicited peak currents ("peak potential", ranging from -45 to -25 mV ) was obtained from this protocol and used in subsequent protocols. Series resistance was also monitored with a 5 ms depolarizing pulse to -105 mV immediately before the test pulse to ensure that this variable was relatively constant and any changes in peak current levels were not due to significant changes in $R_{s}$. Effects of saturating concentrations of mAChR agonist ( 1 mM CCh ) on stable T-type currents were then investigated using steps to peak potential every 5 seconds ( 0.2 Hz ) from a holding potential of -110 mV . These depolarizing steps were 80 ms in duration for $\mathrm{Ca}_{\mathrm{v}} 3.1$ and $\mathrm{Ca}_{\mathrm{v}} 3.2$, and 200 ms in duration for $\mathrm{Ca}_{\mathrm{v}} 3.3$. The -140 mV prepulse protocol for $\mathrm{Ca}_{\mathrm{v}} 3.3$ included a 1 second prepulse to -140 mV to remove any accumulated channel inactivation. To quantify the percent of channel inhibition, stimulation, or washout during CCh or control solution perfusion, the peak current magnitude at equilibrium was averaged (2-5 values). When distinct effects were observed (i.e. - stimulation versus no effect of M1 on $\mathrm{Ca}_{\mathrm{v}} 3.1$ currents) all cells displaying a $>10 \%$ modulating effect with a clear exponential time course were grouped into one group, while the rest of the cells were grouped into the "no effect" group.

Current-voltage relationships were fitted with the modified Boltzmann equation: $I=\left[G_{\max } *\left(V_{\mathrm{m}}-\right.\right.$ $\left.\left.E_{\mathrm{rev}}\right)\right] /\left[1+\exp \left(\left\{V_{\mathrm{m}}-V_{0.5 \mathrm{a}}\right\} / k_{\mathrm{a}}\right)\right]$, where $V_{\mathrm{m}}$ is the test potential, $V_{0.5 \mathrm{a}}$ is the half-activation potential, $E_{\mathrm{rev}}$ is the extrapolated reversal potential, $G_{\max }$ is the maximum slope conductance, and $k_{\mathrm{a}}$ reflects the slope of the activation curve. Data from CCh concentration-response studies were fitted with the equation $y=\left[\left(A_{1}-\right.\right.$ $\left.\left.A_{2}\right) /\left\{1+\left(x / x_{0}\right)^{P}\right\}+A_{2}\right]$ where $A_{1}$ is initial amplitude $(=0)$ and $A_{2}$ is final block value, $x_{0}$ is $\mathrm{IC}_{50}$ (concentration causing 50\% inhibition of currents), and $P$ gives a measure of the steepness of the curve. The activation and inactivation rates during steps to peak potential were well described by single exponential curves to give $\tau_{\text {act }}$ and $\tau_{\text {inact }}$ values, respectively.

Statistical significance was tested with Student's T-tests with significance being determined at a confidence interval of $\mathrm{p}<0.02$.

### 2.4.4 Solutions, drugs, and perfusion

A 25 mM stock of $\beta$-Escin (in $\mathrm{dH}_{2} \mathrm{O}$ ) was prepared fresh, with dilution to working stocks in intracellular solution. Carbachol was added directly to the extracellular recording solution. Wortmannin, okadaic acid, genistein, and H9 were all obtained from Tocris Cookson (Ellisville, MO). Rp-cAMPs was obtained from BioMol International (Plymouth Meeting, PA). BAPTA-AM was obtained from Molecular Probes (Oregon, USA). Unless otherwise stated, all other drugs were obtained from Sigma-Aldrich (St. Louis, MO). Drugs were dissolved in either $\mathrm{dH}_{2} \mathrm{O}$ or DMSO, according to
manufacturer's solubility data. The highest concentration of DMSO in the recording solution did not exceed $0.1 \%$, a concentration that did not detectably affect T-type properties (data not shown). Gravitydriven perfusion occurred at a rate of approximately $400 \mu 1 /$ minute, and the outputs of the manifold were placed within close proximity of the cell, resulting in the cell being bathed in new solutions with minimal delay (within 1 sec ).

### 2.5 Acknowledgements

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### 2.6 References

Allen, T.G., and Brown, D.A. (1993). M2 muscarinic receptor-mediated inhibition of the $\mathrm{C} 22+$ current in rat magnocellular cholinergic basal forebrain neurones. J Physiol 466, 173-189.

Anderson, M.P., Mochizuki, T., Xie, J., Fischler, W., Manger, J.P., Talley, E.M., Scammell, T.E., and Tonegawa, S. (2005). Thalamic Cav3.1 T-type Ca2+ channel plays a crucial role in stabilizing sleep. Proc Natl Acad Sci U S A 102, 1743-1748.

Bannister, R.A., Melliti, K., and Adams, B.A. (2002). Reconstituted slow muscarinic inhibition of neuronal (Ca(v)1.2c) L-type Ca2+ channels. Biophys J 83, 3256-3267.

Bian, J., Cui, J., and McDonald, T.V. (2001). HERG K(+) channel activity is regulated by changes in phosphatidyl inositol 4,5-bisphosphate. Circ Res 89, 1168-1176.

Bourinet, E., Alloui, A., Monteil, A., Barrere, C., Couette, B., Poirot, O., Pages, A., McRory, J., Snutch, T.P., Eschalier, A., and Nargeot, J. (2005). Silencing of the Cav3.2 T-type calcium channel gene in sensory neurons demonstrates its major role in nociception. Embo J 24, 315-324.

Castillo, P.E., Carleton, A., Vincent, J.D., and Lledo, P.M. (1999). Multiple and opposing roles of cholinergic transmission in the main olfactory bulb. J Neurosci 19, 9180-9191.

Chemin, J., Monteil, A., Perez-Reyes, E., Bourinet, E., Nargeot, J., and Lory, P. (2002). Specific contribution of human T-type calcium channel isotypes (alpha(1G), alpha(1H) and alpha(1I)) to neuronal excitability. J Physiol 540, 3-14.

Chemin, J., Monteil, A., Perez-Reyes, E., Nargeot, J., and Lory, P. (2001). Direct inhibition of T-type calcium channels by the endogenous cannabinoid anandamide. Embo J 20, 7033-7040.

Chemin, J., Traboulsie, A., and Lory, P. (2006). Molecular pathways underlying the modulation of Ttype calcium channels by neurotransmitters and hormones. Cell Calcium 40, 121-134.

Chevalier, M., Lory, P., Mironneau, C., Macrez, N., and Quignard, J.F. (2006). T-type CaV3.3 calcium channels produce spontaneous low-threshold action potentials and intracellular calcium oscillations. Eur J Neurosci 23, 2321-2329.

Christie, B.R., Eliot, L.S., Ito, K., Miyakawa, H., and Johnston, D. (1995). Different Ca2+ channels in soma and dendrites of hippocampal pyramidal neurons mediate spike-induced Ca2+ influx. J Neurophysiol 73, 2553-2557.

Cribbs, L.L., Lee, J.H., Yang, J., Satin, J., Zhang, Y., Daud, A., Barclay, J., Williamson, M.P., Fox, M., Rees, M., and Perez-Reyes, E. (1998). Cloning and characterization of alpha1H from human heart, a member of the T-type Ca2+ channel gene family. Circ Res 83, 103-109.

DePuy, S.D., Yao, J., Hu, C., McIntire, W., Bidaud, I., Lory, P., Rastinejad, F., Gonzalez, C., Garrison, J.C., and Barrett, P.Q. (2006). The molecular basis for T-type Ca2+ channel inhibition by G protein beta2gamma2 subunits. Proc Natl Acad Sci U S A 103, 14590-14595.

Destexhe, A., and Sejnowski, T.J. (2003). Interactions between membrane conductances underlying thalamocortical slow-wave oscillations. Physiol Rev 83, 1401-1453.

Dippel, E., Kalkbrenner, F., Wittig, B., and Schultz, G. (1996). A heterotrimeric G protein complex couples the muscarinic ml receptor to phospholipase C-beta. Proc Natl Acad Sci U S A 93, 1391-1396.

Dolphin, A.C. (2003). G protein modulation of voltage-gated calcium channels. Pharmacol Rev 55, 607627.

Egger, V., Svoboda, K., and Mainen, Z.F. (2003). Mechanisms of lateral inhibition in the olfactory bulb: efficiency and modulation of spike-evoked calcium influx into granule cells. J Neurosci 23, 7551-7558.

Egger, V., Svoboda, K., and Mainen, Z.F. (2005). Dendrodendritic synaptic signals in olfactory bulb granule cells: local spine boost and global low-threshold spike. J Neurosci 25, 3521-3530.

Fisher, R., and Johnston, D. (1990). Differential modulation of single voltage-gated calcium channels by cholinergic and adrenergic agonists in adult hippocampal neurons. J Neurophysiol 64, 1291-1302.

Fraser, D.D., and MacVicar, B.A. (1991). Low-threshold transient calcium current in rat hippocampal lacunosum-moleculare interneurons: kinetics and modulation by neurotransmitters. J Neurosci 11, 28122820.

Gamper, N., Reznikov, V., Yamada, Y., Yang, J., and Shapiro, M.S. (2004). Phosphatidylinositol [correction] 4,5-bisphosphate signals underlie receptor-specific Gq/11-mediated modulation of N-type Ca2+ channels. J Neurosci 24, 10980-10992.

Hamid, J., Peloquin, J.B., Monteil, A., and Zamponi, G.W. (2006). Determinants of the differential gating properties of Cav3.1 and Cav3.3 T-type channels: a role of domain IV? Neuroscience 143, 717728.

Heximer, S.P., Watson, N., Linder, M.E., Blumer, K.J., and Hepler, J.R. (1997). RGS2/G0S8 is a selective inhibitor of Gqalpha function. Proc Natl Acad Sci U S A 94, 14389-14393.

Hildebrand, M.E., David, L.S., and Snutch, T.P. (2005). Differential modulation of T-type calcium channels by metabotropic glutamate receptors. In Biophysical Society-49th Annual Meeting (Long Beach, California).

Hildebrand, M.E., and Snutch, T.P. (2006). Contributions of T-type calcium channels to the pathophysiology of pain signaling. Drug Discovery Today: Disease Mechanisms 3, 335-341.

Hogger, P., Shockley, M.S., Lameh, J., and Sadee, W. (1995). Activating and inactivating mutations in N - and C-terminal i3 loop junctions of muscarinic acetylcholine Hm1 receptors. J Biol Chem 270, 74057410.

Huguenard, J.R., and Prince, D.A. (1992). A novel T-type current underlies prolonged $\mathrm{Ca}(2+$ )-dependent burst firing in GABAergic neurons of rat thalamic reticular nucleus. J Neurosci 12, 3804-3817.

Joksovic, P.M., Bayliss, D.A., and Todorovic, S.M. (2005). Different kinetic properties of two T-type $\mathrm{Ca} 2+$ currents of rat reticular thalamic neurones and their modulation by enflurane. J Physiol 566, 125142.

Joksovic, P.M., Nelson, M.T., Jevtovic-Todorovic, V., Patel, M.K., Perez-Reyes, E., Campbell, K.P., Chen, C.C., and Todorovic, S.M. (2006). CaV3.2 is the major molecular substrate for redox regulation of T-type Ca2+ channels in the rat and mouse thalamus. J Physiol 574, 415-430.

Kammermeier, P.J., Ruiz-Velasco, V., and Ikeda, S.R. (2000). A voltage-independent calcium current inhibitory pathway activated by muscarinic agonists in rat sympathetic neurons requires both Galpha $\mathrm{q} / 11$ and Gbeta gamma. J Neurosci 20, 5623-5629.

Khosravani, H., Altier, C., Simms, B., Hamming, K.S., Snutch, T.P., Mezeyova, J., McRory, J.E., and Zamponi, G.W. (2004). Gating effects of mutations in the Cav3.2 T-type calcium channel associated with childhood absence epilepsy. J Biol Chem 279, 9681-9684.

Khosravani, H., Bladen, C., Parker, D.B., Snutch, T.P., McRory, J.E., and Zamponi, G.W. (2005). Effects of Cav3.2 channel mutations linked to idiopathic generalized epilepsy. Ann Neurol 57, 745-749.

Kim, D., Song, I., Keum, S., Lee, T., Jeong, M.J., Kim, S.S., McEnery, M.W., and Shin, H.S. (2001). Lack of the burst firing of thalamocortical relay neurons and resistance to absence seizures in mice lacking alpha(1G) T-type $\mathrm{Ca}(2+$ ) channels. Neuron 31, 35-45.

Lee, J.H., Daud, A.N., Cribbs, L.L., Lacerda, A.E., Pereverzev, A., Klockner, U., Schneider, T., and Perez-Reyes, E. (1999). Cloning and expression of a novel member of the low voltage-activated T-type calcium channel family. J Neurosci 19, 1912-1921.

Levey, A.I., Edmunds, S.M., Heilman, C.J., Desmond, T.J., and Frey, K.A. (1994). Localization of muscarinic m 3 receptor protein and M3 receptor binding in rat brain. Neuroscience 63, 207-221.

Levey, A.I., Edmunds, S.M., Koliatsos, V., Wiley, R.G., and Heilman, C.J. (1995). Expression of m1-m4 muscarinic acetylcholine receptor proteins in rat hippocampus and regulation by cholinergic innervation. J Neurosci 15, 4077-4092.

McKay, B.E., McRory, J.E., Molineux, M.L., Hamid, J., Snutch, T.P., Zamponi, G.W., and Turner, R.W. (2006). Cav3 T-type calcium channel isoforms differentially distribute to somatic and dendritic compartments in rat central neurons. Eur J Neurosci 24, 2581-2594.

McRory, J.E., Santi, C.M., Hamming, K.S., Mezeyova, J., Sutton, K.G., Baillie, D.L., Stea, A., and Snutch, T.P. (2001). Molecular and functional characterization of a family of rat brain T-type calcium channels. J Biol Chem 276, 3999-4011.

Mittman, S., Guo, J., Emerick, M.C., and Agnew, W.S. (1999). Structure and alternative splicing of the gene encoding alpha1I, a human brain T calcium channel alpha1 subunit. Neurosci Lett 269, 121-124.

Monteil, A., Chemin, J., Leuranguer, V., Altier, C., Mennessier, G., Bourinet, E., Lory, P., and Nargeot, J. (2000). Specific properties of T-type calcium channels generated by the human alpha 1I subunit. J Biol Chem 275, 16530-16535.

Murbartian, J., Arias, J.M., Lee, J.H., Gomora, J.C., and Perez-Reyes, E. (2002). Alternative splicing of the rat $\mathrm{Ca}(\mathrm{v}) 3.3 \mathrm{~T}$-type calcium channel gene produces variants with distinct functional properties(1). FEBS Lett 528, 272-278.

Murbartian, J., Arias, J.M., and Perez-Reyes, E. (2004). Functional impact of alternative splicing of human T-type Cav3.3 calcium channels. J Neurophysiol 92, 3399-3407.

Park, J.Y., Kang, H.W., Moon, H.J., Huh, S.U., Jeong, S.W., Soldatov, N.M., and Lee, J.H. (2006). Activation of protein kinase C augments T-type $\mathrm{Ca} 2+$ channel activity without changing channel surface density. J Physiol 577, 513-523.

Peloquin, J.B., Khosravani, H., Barr, W., Bladen, C., Evans, R., Mezeyova, J., Parker, D., Snutch, T.P., McRory, J.E., and Zamponi, G.W. (2006). Functional analysis of Ca3.2 T-type calcium channel mutations linked to childhood absence epilepsy. Epilepsia 47, 655-658.

Pemberton, K.E., Hill-Eubanks, L.J., and Jones, S.V. (2000). Modulation of low-threshold T-type calcium channels by the five muscarinic receptor subtypes in NIH 3 T3 cells. Pflugers Arch 440, 452461.

Perez-Reyes, E., Cribbs, L.L., Daud, A., Lacerda, A.E., Barclay, J., Williamson, M.P., Fox, M., Rees, M., and Lee, J.H. (1998). Molecular characterization of a neuronal low-voltage-activated T-type calcium channel. Nature 391, 896-900.

Plummer, K.L., Manning, K.A., Levey, A.I., Rees, H.D., and Uhlrich, D.J. (1999). Muscarinic receptor subtypes in the lateral geniculate nucleus: a light and electron microscopic analysis. J Comp Neurol 404, 408-425.

Rhee, S.G., and Bae, Y.S. (1997). Regulation of phosphoinositide-specific phospholipase C isozymes. J Biol Chem 272, 15045-15048.

Santi, C.M., Cayabyab, F.S., Sutton, K.G., McRory, J.E., Mezeyova, J., Hamming, K.S., Parker, D., Stea, A., and Snutch, T.P. (2002). Differential inhibition of T-type calcium channels by neuroleptics. J Neurosci 22, 396-403.

Schwarz, R.D., Davis, R.E., Jaen, J.C., Spencer, C.J., Tecle, H., and Thomas, A.J. (1993).
Characterization of muscarinic agonists in recombinant cell lines. Life Sci 52, 465-472.
Suh, B.C., and Hille, B. (2005). Regulation of ion channels by phosphatidylinositol 4,5-bisphosphate. Curr Opin Neurobiol 15, 370-378.

Suh, B.C., Horowitz, L.F., Hirdes, W., Mackie, K., and Hille, B. (2004). Regulation of KCNQ2/KCNQ3 current by G protein cycling: the kinetics of receptor-mediated signaling by Gq. J Gen Physiol 123, 663683.

Talley, E.M., Cribbs, L.L., Lee, J.H., Daud, A., Perez-Reyes, E., and Bayliss, D.A. (1999). Differential distribution of three members of a gene family encoding low voltage-activated (T-type) calcium channels. J Neurosci 19, 1895-1911.

Thompson, S.M., and Wong, R.K. (1991). Development of calcium current subtypes in isolated rat hippocampal pyramidal cells. J Physiol 439, 671-689.

Tombler, E., Cabanilla, N.J., Carman, P., Permaul, N., Hall, J.J., Richman, R.W., Lee, J., Rodriguez, J., Felsenfeld, D.P., Hennigan, R.F., and Diverse-Pierluissi, M.A. (2006). G protein-induced trafficking of voltage-dependent calcium channels. J Biol Chem 281, 1827-1839.

Tsakiridou, E., Bertollini, L., de Curtis, M., Avanzini, G., and Pape, H.C. (1995). Selective increase in Ttype calcium conductance of reticular thalamic neurons in a rat model of absence epilepsy. J Neurosci 15, 3110-3117.

Vitko, I., Chen, Y., Arias, J.M., Shen, Y., Wu, X.R., and Perez-Reyes, E. (2005). Functional characterization and neuronal modeling of the effects of childhood absence epilepsy variants of CACNA1H, a T-type calcium channel. J Neurosci 25, 4844-4855.

Wan, X., Desilets, M., Soboloff, J., Morris, C., and Tsang, B.K. (1996). Muscarinic activation inhibits Ttype Ca2+ current in hen granulosa cells. Endocrinology 137, 2514-2521.

Wei, J., Walton, E.A., Milici, A., and Buccafusco, J.J. (1994). m1-m5 muscarinic receptor distribution in rat CNS by RT-PCR and HPLC. J Neurochem 63, 815-821.

Welsby, P.J., Wang, H., Wolfe, J.T., Colbran, R.J., Johnson, M.L., and Barrett, P.Q. (2003). A mechanism for the direct regulation of T-type calcium channels by Ca2+/calmodulin-dependent kinase II. J Neurosci 23, 10116-10121.

Wolfe, J.T., Wang, H., Howard, J., Garrison, J.C., and Barrett, P.Q. (2003). T-type calcium channel regulation by specific G-protein betagamma subunits. Nature 424, 209-213.

Wu, L., Bauer, C.S., Zhen, X.G., Xie, C., and Yang, J. (2002). Dual regulation of voltage-gated calcium channels by PtdIns(4,5)P2. Nature 419, 947-952.

Yunker, A.M., Sharp, A.H., Sundarraj, S., Ranganathan, V., Copeland, T.D., and McEnery, M.W. (2003). Immunological characterization of T-type voltage-dependent calcium channel CaV3.1 (alpha 1G) and CaV3.3 (alpha 1I) isoforms reveal differences in their localization, expression, and neural development. Neuroscience 117, 321-335.

Zamponi, G.W., and Snutch, T.P. (2002). Modulating modulation: crosstalk between regulatory pathways of presynaptic calcium channels. Mol Interv 2, 476-478.

## 3 FUNCTIONAL COUPLING BETWEEN MGLUR1 AND CAv3.1 T-TYPE CALCIUM CHANNELS ENHANCES CEREBELLAR PURKINJE CELL EXCITABILITY AND LOCAL SIGNALING*

### 3.1 Introduction

T-type $\mathrm{Ca}^{2+}$ channels are expressed in a wide range of tissues, including in the nervous, cardiovascular and endocrine systems (Perez-Reyes, 2003). At the molecular level the T-type $\mathrm{Ca}^{2+}$ channel family is composed of three main subtypes $\left(\mathrm{Ca}_{v} 3.1, \mathrm{Ca}_{v} 3.2\right.$, and $\left.\mathrm{Ca}_{v} 3.3\right)$ characterized by similar low thresholds of activation, but with distinct biophysical and modulatory properties and unique cellular and subcellular expression patterns (Talley et al., 1999). Recent knock-down studies suggest that individual T-type channel subtypes might play specific physiological roles. For example, Cav 3.2 KO mice show developmental defects in coronary arteries (Chen et al., 2003) while siRNA-mediated $\mathrm{Ca}_{\mathrm{v}} 3.2$ silencing in sensory neurons has antinociceptive effects (Bourinet et al., 2005). Furthermore, in Cav 3.1 KO mice, the typical burst firing mode of thalamic relay cells is absent and defects in sleep behavior are observed (Anderson et al., 2005).

T-type currents play a critical role in the soma and dendrites of neurons in initiating dendritic boosting and $\mathrm{Ca}^{2+}$ spikes (Egger et al., 2005), in altering neuronal excitability and firing patterns (Crunelli et al., 2006; Kim et al., 2001) and in modulating synaptic integration and plasticity (Christie et al., 1997; Ikeda et al., 2003). Each T-type $\mathrm{Ca}^{2+}$ channel isoform likely plays different roles in these effects depending upon the combination of its cellular and subcellular localization (McKay et al., 2006) and unique biophysical properties (Chemin et al., 2002; Kozlov et al., 1999; McRory et al., 2001). The differential modulation of T-type $\mathrm{Ca}^{2+}$ channel isoforms may constitute an additional substrate of functional specialization and heterogeneity. While the $\mathrm{Ca}_{\mathrm{v}} 3.2$ isoform is specifically modulated by $\mathrm{G} \beta_{2} \gamma$ proteins, CAMKII, and redox changes (Joksovic et al., 2006; Welsby et al., 2003; Wolfe et al., 2003), $\mathrm{Ca}_{\mathrm{v}} 3.2$ and $\mathrm{Ca}_{\mathrm{v}} 3.1 / \mathrm{Ca}_{\mathrm{v}} 3.3$ are modulated in opposite directions by lysophosphatidic acid (Iftinca et al., 2007). $\mathrm{Ga}_{q / 11}$-coupled mAChRs inhibit $\mathrm{Ca}_{\mathrm{v}} 3.3$ channels but not $\mathrm{Ca}_{\mathrm{v}} 3.1$ or $\mathrm{Ca}_{\mathrm{v}} 3.2$ channels (Hildebrand et al., 2007). Overall, these results suggest that subtype-specific modulation of T-type $\mathrm{Ca}^{2+}$ channels may participate in system-specific specializations, although explorations into these contributions in native neuronal systems is thus far lacking.

Cerebellar PCs express the $\mathrm{Ca}_{\mathrm{v}} 3.1$ and $\mathrm{Ca}_{\mathrm{v}} 3.3$ T-type $\mathrm{Ca}^{2+}$ channel isoforms (Hartmann et al., 2004; Isope and Murphy, 2005; McKay et al., 2006). We recently combined whole-cell recordings with

[^1]$\mathrm{Ca}^{2+}$ imaging to demonstrate the presence of $\mathrm{Ca}_{\mathrm{v}} 3.1$-like T-type currents/transients within both spines and dendrites of PCs (Isope and Murphy, 2005). Although their exact functions in PCs remains to be elucidated, T-type currents have been linked to the generation of dendritic low-threshold spikes and in altering interspike and interburst intervals during spontaneous dendritic burst firing (Pouille et al., 2000; Swensen and Bean, 2003; Womack and Khodakhah, 2004). PCs also show the highest relative expression of the G-protein $\mathrm{G} \mathrm{\alpha}_{q / 11}$ in the CNS (Hartmann et al., 2004; Tanaka et al., 2000). In PCs, $\mathrm{G} \alpha_{q / 11}$ is linked to the mGluR1 subtype of metabotropic glutamate receptors. mGluR1 receptors play a central role in the induction of short-term and long-term plasticity at the PF synapse and also control cerebellar motor learning (Aiba et al., 1994; Conquet et al., 1994; Finch and Augustine, 1998; Ichise et al., 2000; Kishimoto et al., 2002; Takechi et al., 1998). Activation of mGluR1 causes PLC translocation and $\mathrm{IP}_{3}$-mediated local $\mathrm{Ca}^{2+}$ release in spines and dendrites (Finch and Augustine, 1998; Takechi et al., 1998). mGluR1 has also been shown to affect intracellular $\mathrm{Ca}^{2+}$ levels within signaling domains of PC dendrites by activating a sEPSC (potentially TRPC1) (Kim et al., 2003; Kitano et al., 2003; Tempia et al., 2001).

Here, we show that mGluR1a receptor activation differentially modulates recombinant T-type isoforms, potentiating $\mathrm{Ca}_{\mathrm{v}} 3.1$ and $\mathrm{Ca}_{\mathrm{v}} 3.2$ while inhibiting $\mathrm{Ca}_{\mathrm{v}} 3.3$. In PCs of acute rodent slices we characterize an mGluR1-mediated potentiation of Cav 3.1 T-type currents that we find occurs through a G-protein and protein tyrosine phosphatase-dependent pathway, is localized to dendritic compartments, and that alters PC excitability. Finally, we show that this potentiation of $\mathrm{Ca}_{\mathrm{v}} 3.1$ by mGluR 1 can be triggered by trains of PF stimulation, suggesting a new potential role for the Cav 3.1 T -type $\mathrm{Ca}^{2+}$ channel isoform in cerebellar synaptic integration.

### 3.2 Results

### 3.2.1 Subtype-specific modulation of recombinant T-type calcium channels by mGluR1a activation

We initially tested whether activation of mGluR1a, a GPCR coupled to $\mathrm{G} \alpha_{q / 11}$, could modulate T-type $\mathrm{Ca}^{2+}$ channels exogenously expressed in HEK cells. Transfection of mGluR1a into HEK 293 cell lines that stably expressed each of the three recombinant rat brain T-type isoforms showed a distinct pattern of modulation; activation of mGluR1a with $100 \mu \mathrm{M}$ glutamate caused a potentiation of $\mathrm{Ca}_{\mathrm{v}} 3.1$ and $\mathrm{Ca}_{\mathrm{v}} 3.2$ currents and an inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.3$ currents (Fig. 3.1). The time course of the potentiation of Ca 3.1 and Ca 3.2 currents was slow $\left(\mathrm{Ca}_{\mathrm{v}} 3.1, \tau_{\text {onset }}=95+/-15 \mathrm{~s}, \mathrm{n}=6\right.$; Cav3.2, $\left.\tau_{\text {onset }}=225+/-22 \mathrm{~s}, \mathrm{n}=5\right)$ and included a "lag" period compared to the rapid onset inhibition of the $\mathrm{Ca}_{\mathrm{v}} 3.3$ currents ( $\tau_{\text {onset }}=16+/-2$ $\mathrm{s}, \mathrm{n}=14$ ), suggesting that the intracellular pathway mediating the two effects could be different (Fig. 3.1).


Figure 3.1 - Recombinant T-type calcium channels are differentially modulated by mGluR1a receptors.
A-C) Left Panels: Representative voltage-clamped current traces during depolarizing pulses from a holding potential of -110 mV to -30 mV showing potentiation of $\mathrm{Ca}_{\mathrm{v}} 3.1$ (A) and $\mathrm{Ca}_{\mathrm{v}} 3.2$ (B) currents and inhibition of $\mathrm{Ca}_{v} 3.3$ (C) currents by activation of mGluR1a receptors with $100 \mu \mathrm{M}$ glutamate. Open shapes indicate traces during control perfusion while closed shapes indicate traces during $100 \mu \mathrm{M}$ glutamate perfusion. Right Panels: Normalized peak current levels during perfusion of control recording solution ( $2 \mathrm{mM} \mathrm{Ca}^{2+}$ ) followed by $100 \mu \mathrm{M}$ glutamate for $\mathrm{Ca}_{\mathrm{v}} 3.1$ (A), $\mathrm{Ca}_{\mathrm{v}} 3.2$ (B), and $\mathrm{Ca}_{\mathrm{v}} 3.3$ (C) currents. The potentiation shown for $\mathrm{Ca}_{\vee} 3.1$ (A) and $\mathrm{Ca}_{v} 3.2$ (B) channels was observed in $\sim 30 \%$ of cells tested, while the mGluR1a-induced inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.3$ currents was observed in $\sim 70 \%$ of cells. mGluR1a activation caused an average potentiation of $\mathrm{Ca}_{\mathrm{v}} 3.1$ currents by $29.6 \%(+/-5.8 \% ; \mathrm{n}=8)$, potentiation of $\mathrm{Ca}_{\mathrm{v}} 3.2$ currents by $41.8 \%(+/-8.8 \% ; \mathrm{n}=6)$, and inhibition of Ca 3.3 currents by $28.7 \%$ (+/$1.9 \%, \mathrm{n}=33$ ). For $\mathrm{Ca}_{\mathrm{v}} 3.1$ and $\mathrm{Ca}_{\mathrm{v}} 3.2$ currents, glutamate had no effect on all excluded cells, while for $\mathrm{Ca}_{\mathrm{v}} 3.3100 \mu \mathrm{M}$ glutamate caused either no effect or inhibition followed by a recovery in the cells not shown (data not shown, see section 3.4 for further details).
$\mathrm{Ca}_{\mathrm{v}} 3.1$ (Fig. 3.1A) and $\mathrm{Ca}_{\mathrm{v}} 3.2$ (Fig. 3.1B) current kinetics were not significantly ( $\mathrm{p}>0.05$ ) affected during the mGluR1a-induced potentiation, while the inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.3$ by mGluR1a was accompanied by a robust increase in activation and inactivation kinetics (Fig. 3.1C; for $\mathrm{Ca}_{\mathrm{v}} 3.3$ : Control, $\tau_{\text {act }}=6.4+/-0.4 \mathrm{~ms}, \mathrm{n}=25, \tau_{\text {inact }}=76+/-8 \mathrm{~ms}, \mathrm{n}=13 ; 100 \mu M$ Glutamate $\tau_{\text {act }}=4.4+/-0.3 \mathrm{~ms}, \mathrm{n}=25, \tau_{\text {inact }}$ $=39+/-5 \mathrm{~ms}, \mathrm{n}=13, \mathrm{p}<0.02$ ). We note that a subset of cells for all three T-type isoforms showed no modulation by mGluR1a, which could be explained by a lack of functional expression of mGluR1a in some transfected cells and/or the heterogeneous efficacy of the downstream signaling cascade.

### 3.2.2 Cavver.1-mediated T-type calcium currents are potentiated by mGluR1 activation in cerebellar Purkinje neurons

We next examined the modulation of T-type currents by mGluR1 in a native system, cerebellar PCs. In PCs, mGluR1 is highly expressed both perisynaptically at PF and at CF contacts (Baude et al., 1993; Lopez-Bendito et al., 2001). Additionally, we have recently shown that T-type channels are expressed in PC dendrites and spines, where they evoke $\mathrm{Ca}^{2+}$ entry upon low threshold depolarization (Isope and Murphy, 2005). In the present study, T-type currents were recorded in whole-cell voltage clamp in PCs prepared from cerebellar slices of male Wistar rats aged P8 to P12. At this age, mGluR1 and T-type currents are both expressed (Isope and Murphy, 2005; Shigemoto et al., 1992) and the dendritic arbour is small enough to alleviate space clamp problems (Roth and Hausser, 2001; Sacco and Tempia, 2002). A combination of pharmacological antagonists were used to isolate T-type currents from potential contamination by $\mathrm{Na}^{+}, \mathrm{K}^{+}$, and HVA $\mathrm{Ca}^{2+}$ currents (as previously described (Isope and Murphy, 2005) and discussed in section 3.4). To ensure the quality of the space-clamp, modulatory effects were studied on T-type currents of moderate amplitude ( $213+/-35 \mathrm{pA}, \mathrm{n}=19$ ) elicited every 10 seconds from a holding potential of -75 mV (with a 500 ms prepulse to -90 mV to remove inactivation) and depolarizing test pulses ranging between -50 mV and -35 mV (Fig. 3.2A-C). Activation of mGluR1 receptors by bath-application of DHPG $(20 \mu \mathrm{M})$ caused a robust and reversible increase in T-type peak current amplitude ( $51+/-7 \%, n=19$; reversed by $84+/-11 \%, n=5$; Fig. 3.2A,B) in all PCs tested. The DHPGinduced potentiation of T-type currents did not involve a significant change in the activation ( $\tau_{\text {act }}$ ) or inactivation ( $\tau_{\text {inact }}$ ) kinetics of the current (Fig. 3.2A, Table 3.1). The increase in T-type currents was not due to changes in passive electrical properties, as $R_{s}$ and leak currents ( $I_{L}$; at $V_{h}=-75 \mathrm{mV}$ ) remained relatively constant between the measured conditions (Control, $\mathrm{R}_{\mathrm{s}}=10.4+/-1.5 \mathrm{M} \Omega, \mathrm{I}_{\mathrm{L}}=-204+/-46 \mathrm{pA}$; DHPG for 2 minutes, $\mathrm{R}_{\mathrm{s}}=10.4+/-1.8 \mathrm{M} \Omega, \mathrm{I}_{\mathrm{L}}=-233+/-49 \mathrm{pA}(\mathrm{n}=9)$ ).

Bath application of DHPG tonically activates mGluR1 receptors to produce robust and reproducible maximal effects, but this form of receptor activation may lead to receptor desensitization
and does not accurately mimic a physiological activation. To overcome this limitation, we used brief puffs of DHPG (see section 3.4) previously shown to reproduce the sEPSC responses when mGluR1 was synaptically activated by brief tetanic stimulation of PFs (Tempia et al., 2001). In our experiments, puffing on $100 \mu \mathrm{M}$ DHPG caused a fast potentiation of T-type currents ( $45+/-12 \%, \mathrm{n}=6$ ) that reached a maximal level within 10 seconds (Fig. 3.2D). An increase in leak current (likely due to the activation of sEPSCs) was observed for the first two pulses after the DHPG puff ( $\mathrm{I}_{\mathrm{L}}=-279+/-132 \mathrm{pA}, \mathrm{n}=6$ ) but leak values then returned to control levels ( $\mathrm{I}_{\mathrm{L}}=-171+/-94 \mathrm{pA}, \mathrm{n}=6$ ) and the T-type potentiation remained. Similar to bath application of DHPG (not shown), the DHPG puff-induced increase in T-type currents was reversed (by $94+/-9 \%, \mathrm{n}=6$ ) upon prolonged perfusion of control solution.

Since DHPG activates both mGluR1 and mGluR5 receptors, we assessed the specificity of the effect using co-application of DHPG with a group 1 antagonist ( $500 \mu \mathrm{M}$ MCPG) or LY367385 (100 $\mu \mathrm{M})$, a specific mGluR1 antagonist. In both conditions, the T-type potentiation was completely blocked, indicating that the DHPG-induced potentiation of T-type currents occurs specifically through mGluR1 receptors (Fig. 3.5B,F).

Our exogenous expression experiments in transfected HEK cells indicated that both $\mathrm{Ca}_{v} 3.1$ and $\mathrm{Ca}_{\mathrm{v}} 3.2$ T-type channel isoforms were capable of being upregulated by mGluR1 activation (Fig. 3.1). As PCs appear to express the $\mathrm{Ca}_{\mathrm{v}} 3.1$ and $\mathrm{Ca}_{\mathrm{v}} 3.3 \mathrm{~T}$-type $\mathrm{Ca}^{2+}$ isoforms but not $\mathrm{Ca}_{\mathrm{v}} 3.2$ (Hartmann et al., 2004; Isope and Murphy, 2005; McKay et al., 2006), the observed T-type current upregulation in PCs was predicted to be due to native $\mathrm{Ca}_{\mathrm{v}} 3.1$ channel modulation. To test this notion we examined PCs in $\mathrm{Ca}_{\mathrm{v}} 3.1$ KO mice ( P 8 to P12). Figure 3.2 (E,F) shows that under our measurement conditions PCs from $\mathrm{Ca}_{\mathrm{v}} 3.1$ KO mice completely lacked T-type whole cell currents during depolarizing test pulses to -40 mV that normally induce robust currents in the wt mice ( $\mathrm{Ca}_{\mathrm{v}} 3.1 \mathrm{KO}=12+/-16 \mathrm{pA}, \mathrm{n}=4$; wt $=1280+/-85 \mathrm{pA}$ at $40 \mathrm{mV}, \mathrm{n}=3$ ). Thus, T-type currents of immature rodent PCs are principally mediated by $\mathrm{Ca}_{\mathrm{v}} 3.1$ channels.

We note that R -type $\mathrm{Ca}^{2+}$ currents show some voltage-dependent and kinetic properties similar to those for $\mathrm{Ca}_{\mathrm{v}} 3.1$ and that R-type channels may also be expressed in PCs (Meacham et al., 2003). In order to rule out the possibility that in wt mice the potentiating effect of mGluR1 activation might in part be carried by R-type $\mathrm{Ca}^{2+}$ currents $\left(\mathrm{Ca}_{\mathrm{v}} 2.3\right)$ we examined a strain of $\mathrm{Ca}_{\mathrm{v}} 2.3 \mathrm{KO}$ mice. Figure 3.2F shows that the DHPG-induced $\mathrm{Ca}^{2+}$ current potentiation in PCs was similar in $\mathrm{Ca}_{\mathrm{v}} 2.3 \mathrm{KO}$ and wt mice (Fig. 3.2F). Taken together, our findings suggest that mGluR1 receptor activation in PCs specifically and selectively potentiates T-type currents mediated by $\mathrm{Ca}_{\mathrm{v}} 3.1$ channels.


Figure 3.2 - T-type calcium channels in cerebellar PCs are reversibly potentiated by mGluR1 activation.
A) Representative voltage-clamped current traces for rat PC T-type currents during depolarizing pulses from -90 mV to -43 mV before (1), during (2), and after (3) activation of endogenous mGluR1 receptors with $20 \mu \mathrm{M}$ DHPG. B) Normalized peak current time course for the cell shown in A demonstrating the reversibility of DHPG-induced potentiation. C) Average normalized peak T-type current time course during control perfusion followed by bath perfusion of $20 \mu \mathrm{M}$ DHPG. T-type currents increased by $51 \%$ $+/-7 \%(\mathrm{n}=19)$ after 100 to 120 seconds of DHPG application. D) Micropressure ( $50 \mathrm{~ms}, 10$ PSI) pulses of DHPG $(100 \mu \mathrm{M})$ through a puff pipette triggered a rapid potentiation of T-type current amplitude by $45 \%+/-12 \%(n=6)$. E) Representative current traces during depolarizations from -80 mV to -40 mV in both wt (black trace) and Cav 3.1 -/- (grey trace) mice. F) Left: Quantification of peak PC T-type currents in Cav $3.1-/-$ mice $(\mathrm{n}=4)$ compared to wt mice $(\mathrm{n}=4)$ during depolarizing steps to -40 mV . Right: DHPG causes robust and equal T-type potentiation in wt mice ( $\mathrm{n}=3$ ) and $\mathrm{Ca}_{\mathrm{v}} 2.3-/-$ mice $(\mathrm{n}=4)$.

Table 3.2 - Effects of DHPG on Purkinje Cell T-Type Biophysical Properties

|  | Control | DHPG |
| :---: | :---: | :---: |
| T-type Properties |  |  |
| $\mathrm{V}_{50-\text {-activation }}(\mathrm{mV})$ | $-31.3+/-0.6, \mathrm{n}=7$ | $-33.7+/-0.7, \mathrm{n}=7$ * |
| k, activation | $-4.5+/-0.1, \mathrm{n}=7$ | $-4.7+/-0.1, \mathrm{n}=7$ |
|  |  |  |
| $\mathrm{~V}_{50-\text {-inactivation }}(\mathrm{mV})$ | $-64.5+/-1.8, \mathrm{n}=6$ | $-67.8+/-1.8, \mathrm{n}=6$ |
| k, inactivation | $6.6+/-1.0, \mathrm{n}=6$ | $6.5+/-0.8, \mathrm{n}=6$ |
|  |  |  |
| $\tau_{\text {activation }}(\mathrm{ms})$ | $7.0+/-0.5, \mathrm{n}=16$ | $7.7+/-0.8, \mathrm{n}=16$ |
| $\tau_{\text {inactivation }}(\mathrm{ms})$ | $36.9+/-2.9, \mathrm{n}=18$ | $31.5+/-2.2, \mathrm{n}=18$ |
| $\tau_{\text {deactivation }}(\mathrm{ms})$ | $3.2+/-0.5, \mathrm{n}=7$ | $3.1+/-0.4, \mathrm{n}=7$ |

* $\mathrm{p}<0.02$
- DHPG indicates 20 to $30 \mu \mathrm{M}$ DHPG
$-\tau_{\text {activation }}$ and $\tau_{\text {inactivation }}$ determined at T-type test pulse potential
- $\tau_{\text {deactivation }}$ value for repolarization to -100 mV


### 3.2.3 mGluR1 potentiates T-type currents through an increase in maximal current and a shift in the voltage-dependence of activation

The effect of mGluR1 activation (via DHPG bath perfusion) on the voltage-dependence of Ttype activation was studied using a series of test depolarizations ranging from -70 mV to -5 mV . In order to minimize the effects of contaminating $\mathrm{HVA} \mathrm{Ca}^{2+}$ currents (especially P-type) and $\mathrm{K}^{+}$currents in this broader voltage range, a stronger pharmacological cocktail was used that included $300 \mu \mathrm{M} \mathrm{Cd}^{2+}, 10-$ $20 \mathrm{mM} \mathrm{TEA}^{+}$, and $5-10 \mathrm{mM} 4$-AP. Figure 3.3A shows a representative cell where mGluR1 activation with DHPG $(30 \mu \mathrm{M})$ caused an increase in the remaining rat T-type currents at all potentials. The DHPG-mediated increase in peak current was significant ( $\mathrm{p}<0.05$ ) at potentials between -45 mV and -20 mV (Fig. 3.3B). DHPG application also caused a significant $(-2.4 \mathrm{mV}, \mathrm{n}=7, \mathrm{p}<0.02)$ shift in the halfactivation potential ( $\mathrm{V}_{50 \mathrm{act}}$ ) of the T-type currents (Fig. 3.3C, Table 3.1) but no significant ( $\mathrm{p}>0.05$ ) effect on the voltage-dependence of T-type steady-state inactivation (Fig. 3.3D) or the voltage dependence of deactivation kinetics (Fig. 3.3E). These results indicate that the DHPG potentiation effect is mediated both by an increase in maximal current and a shift in the voltage-dependence of activation, suggesting a change in open probability and/or conductance of the channels. The strong voltage-dependence of these deactivation kinetics (Randall and Tsien, 1997) combined with the fact that $50 \mu \mathrm{M} \mathrm{Ni}^{2+}$ was included to block R-type currents provide further confirmation that the DHPG-potentiated low threshold currents are due to T-type, and not R-type, channels.

### 3.2.4 mGluR1 activation increases PC excitability via effects on T-type calcium currents

The current-voltage relationships shown in Figure 3.3B indicates that the maximum mGluR1mediated potentiation occurs at potentials between -45 mV and $-40 \mathrm{mV}(110 \%$ increase, $\mathrm{n}=7$ at $-40 \mathrm{mV}$ compared to $22 \%$ increase, $\mathrm{n}=7$ at -20 mV ). These potentials are very close to the somatic threshold for the generation of sodium spikes and we hypothesized that the subthreshold potentiation of T-type currents by mGluR1 could shorten the first spike latency during a depolarizing event. To test this hypothesis, current clamp experiments were performed using the same pharmacological cocktail described in voltage-clamp experiments in order to isolate changes in the rat PC membrane potential that are due to T-type activity. Hyperpolarized initial resting potential values ( $-82.6+/-1.1 \mathrm{mV}, \mathrm{n}=5$ ) were obtained via constant current injection, averaging $-191+/-17 \mathrm{pA}(\mathrm{n}=5)$ to ensure the availability of Ttype currents (Fig. 3.4). In the absence of voltage-gated $\mathrm{Na}^{+}, \mathrm{K}^{+}$, and $\mathrm{HVA} \mathrm{Ca}^{2+}$ currents (validated by voltage-clamp IV curves; data not shown), injection of depolarizing currents (relative to holding currents; averaging $154 \mathrm{pA}+/-9 \mathrm{pA}, \mathrm{n}=5$ ) resulted in T -type-mediated $\mathrm{Ca}^{2+}$ spikes and a sustained plateau potential with minimal repolarization (due to the blockade of $\mathrm{K}^{+}$currents; (Swensen and Bean, 2003) (Fig. 3.4A)).


Figure 3.3 - T-type calcium currents are potentiated by mGluR1 through an increase in maximal current and a small shift in the voltage-dependence of activation.
A) Representative voltage-clamped current traces during depolarizations from -90 mV to potentials ranging from -70 mV to -5 mV before (left) and after (right) mGluR1 was activated with $30 \mu \mathrm{M} \mathrm{DHPG}$, in the presence of a cocktail containing $300 \mu \mathrm{M} \mathrm{Cd}^{2+}, 10-20 \mathrm{mM} \mathrm{TEA}^{+}$, and $5-10 \mathrm{mM} 4$-AP. Recordings were from PCs of P8 to P12 Wistar rats. B-E) Control recordings are represented by black squares while recordings in DHPG are represented by grey circles. B) Normalized IV curve showing that DHPG increases maximal current, resulting in a significant potentiation of T-type currents at potentials between -45 mV and $-20 \mathrm{mV}(\mathrm{n}=7)$. * indicates significance at $\mathrm{p}<0.05$ compared to control current values. C) Normalized conductance curve for PC T-type currents generated by fitting the IV curves in $\mathbf{B}$ with a modified Boltzmann equation (see Methods). DHPG application caused a small, but significant ( $\mathrm{p}<0.02$ ) shift of $\sim 2 \mathrm{mV}$ in the $\mathrm{V}_{50 \mathrm{act}}$ for these currents (Table 1). D) Activation of mGluR1 with $20 \mu \mathrm{M} \mathrm{DHPG}$ had no significant ( $\mathrm{p}>0.05$ ) effect on the steady-state inactivation $\left(\mathrm{V}_{50 \text { inact }}\right)$ of T-type currents within PCs (Table 1). E) Perfusion of $20 \mu \mathrm{M}$ DHPG had no significant ( $\gg 0.05$ ) effects on the kinetics of T-type channel deactivation (Table 1). Inset: Waveform protocol showed on top and representative current traces shown below for a PC under control conditions. For scale bar, X axis $=20 \mathrm{~ms}, \mathrm{Y}$ axis $=200 \mathrm{pA}$.

Bath application of $20 \mu \mathrm{M}$ DHPG significantly ( $\mathrm{p}<0.02$ ) decreased the temporal latency (by 82 $\mathrm{ms}, \mathrm{n}=7$ ) and the voltage threshold (by $6.9 \mathrm{mV}, \mathrm{n}=7$ ) of these T-type-dependent $\mathrm{Ca}^{2+}$ spikes and significantly ( $\mathrm{p}<0.02$ ) increased their maximal slope $\left(\mathrm{dV} / \mathrm{dt}_{\text {max }}\right)$ in a reversible manner (Fig. 3.4A,B,D). The ability of DHPG to increase T-type spike activity by decreasing spike threshold and latency was not due to changes in passive properties, as $\mathrm{R}_{\mathrm{N}}$ and $\mathrm{R}_{\mathrm{s}}$ were not significantly ( $\mathrm{p}>0.05$ ) altered during experiments (data not shown; Fig. 3.4D).

Purkinje cells are known to undergo transitions from a hyperpolarized inactive state to a depolarized state, where they fire spontaneously both in vitro (Williams et al., 2002) and in vivo (Loewenstein et al., 2005). Ih has been shown to prevent PC bistability (Williams et al., 2002) but endogenous conductances promoting the bistable behavior have not been analyzed in detail. T-type currents are ideally suited to boost the transition from hyperpolarized to depolarized potentials (Williams et al., 1997). We conducted experiments, using physiological internal and external solutions, to test the participation of T-type currents in rat PC state transitions. A relatively hyperpolarized resting potential (76.1 mV , Fig. 3.4C,D) was maintained with constant injection of current $(-228+/-28 \mathrm{pA}, \mathrm{n}=8)$ to ensure availability of T-type currents, and an average depolarizing injection of $165+/-25 \mathrm{pA}, \mathrm{n}=8$ induced AP firing during control recordings. Application of $20 \mu \mathrm{M}$ DHPG caused a significant ( $\mathrm{p}<0.05$ ) decrease in the voltage threshold for AP firing by $4.0 \mathrm{mV}(\mathrm{n}=8)$ and decreased the first spike latency by $41 \mathrm{~ms}(\mathrm{n}=8)$ (Fig. 3.4C,D). These DHPG-mediated increases in PC membrane excitability were completely reversed with the co-perfusion of the T-type antagonist mibefradil ( $10 \mu \mathrm{M}$; Fig. 3.4C,D). While relatively high concentrations of antagonists such as mibefradil are required to block T-type currents in slice recordings (as confirmed by voltage-clamp recordings, data not shown), mibefradil's $>100$-fold higher affinity for T-type currents over P-type currents in PCs suggests that T-type $\mathrm{Ca}^{2+}$ currents will be selectively blocked at this concentration (McDonough and Bean, 1998). To support these results, $200 \mu \mathrm{M} \mathrm{Ni}^{2+}$ was also used as a T-type antagonist predicted to have no effect on either P/Q-type $\mathrm{Ca}^{2+}$ channels or voltage-gated $\mathrm{Na}^{+}$channels (Kuo et al., 2004; Zamponi et al., 1996). When co-applied with DHPG, $200 \mu \mathrm{M} \mathrm{Ni}^{2+}$ either reversed the DHPG effect ( $\mathrm{n}=3$ ) as was observed for mibefradil or completely eliminated AP firing in the current injection range used ( $n=3$ ) (data not shown). As observed for the effects of DHPG on Ttype spike thresholds, the effects of DHPG on overall PC excitability were not due to changes in $R_{N}$ or $R_{S}$ (data not shown; Fig. 3.4D). These findings suggest that activation of mGluR1 boosts subthreshold membrane depolarization via an increase in T-type currents, leading to a decrease in the threshold and the latency of the first spike at the soma of the PC.


Figure 3.4 - DHPG lowers the threshold for PC T-type-dependent calcium spikes and AP firing.
A) Top Panels: Current clamp traces from a representative rat PC during hyperpolarizing and depolarizing current injection pulses before (left), during (middle), and after (right) DHPG application. Similar to voltage-clamp experiments, T-type conductances were isolated with 300 nM TTX, $50 \mu \mathrm{M}$ $\mathrm{Ni}^{2+}, 20 \mathrm{mM}$ TEA, $5 \mathrm{mM} 4-\mathrm{AP}$, and $50 \mu \mathrm{M} \mathrm{Cd}^{2+}$ in the external solution and a $\mathrm{Cs}^{+}$-based internal solution. Bottom Panels: Current injection protocol for above traces showing steps to $-100,-50,0,50$, $100,110,120$, and 130 pA . B) Current injection steps from A (to 130 pA , grey traces) that induced T-type-dependent $\mathrm{Ca}^{2+}$ spikes in all conditions. Bath application of $20 \mu \mathrm{M}$ DHPG significantly ( $\mathrm{p}<0.02$ ) decreased both the spike latency and the voltage threshold for T-type-dependent $\mathrm{Ca}^{2+}$ spike activation (see panel D). Washout of DHPG reversed the changes in the latency and voltage threshold of T-typedependent $\mathrm{Ca}^{2+}$ spikes (see panel D). C) Current clamp traces from a representative PC in ACSF solution with no voltage-gated ion channel antagonists during a depolarizing current injection step that elicited AP firing during control recordings (thin line), perfusion of $20 \mu \mathrm{M} \mathrm{DHPG}$ (thick line), and finally co-application of $20 \mu \mathrm{M}$ DHPG with $10 \mu \mathrm{M}$ mibefradil (dashed line). Application of DHPG decreased the latency and voltage threshold for firing of the first AP, which was reversed upon application of the T-type antagonist, mibefradil. D) Top: Table of passive and active properties before (Control), during ( 20 mM DHPG), and after (Wash) perfusion of $20 \mu \mathrm{M} \mathrm{DHPG}$. I Threshold $=$ first current injection step where T-type spikes are observed; V Threshold = voltage of inflection point where T-type spikes are initiated; Spike Latency = time point, from start of depolarizing current step, at which T-type spike inflection point occurs; $\mathrm{dV} / \mathrm{dt}_{\max }=$ maximum slope of T-type spike as determined by differentiation (see section 3.4); $\mathrm{V}_{\text {rest }}=$ membrane potential before depolarizing current step. V threshold, Spike Latency, $\mathrm{dV} / \mathrm{dt}_{\text {max }}$ and $\mathrm{V}_{\text {rest }}$ were measured from a constant depolarizing current injection step that elicited T-type spikes under all conditions for a given cell. Bottom: Table of the same parameters derived for first AP firing in physiological saline, except that the spike latency and V threshold are now calculated from the inflection point of the first AP. * represents significance at $\mathrm{p}<0.02$ compared to control values.

### 3.2.5 mGluR1 potentiates T-type currents through a G-protein-, tyrosine phosphatase-, and calcium- dependent pathway independently of phospholipase $\mathbf{C}$ and its downstream

 effectorsMultiple intracellular pathways are known to be associated with mGluR1 receptors in PCs (Knopfel and Grandes, 2002) and we set out to study the identity of the signaling pathway leading to Ttype current potentiation. Inclusion of 2 mM GDP- $\beta-\mathrm{S}$ in the pipette solution to block G-protein activity ( $\mathrm{G} \alpha$ and $\mathrm{G} \beta \gamma$ ) completely abolished the potentiation of rat T-type currents by DHPG (Fig. 3.5C,F). However, blocking PLC with either $1 \mu \mathrm{M}$ U73122 or $10 \mu \mathrm{M}$ edelfosine, PKC with 1 to $2.5 \mu \mathrm{M}$ staurosporine, or $\mathrm{IP}_{3}$ Rs with $1 \mu \mathrm{M}$ xestospongin C all had no significant ( $\mathrm{p}>0.05$ ) effect on the DHPGmediated increase in T-type currents (Fig. 3.5F). Using a similar approach, Canepari and Ogden showed that activation of sEPSCs by mGluR1 was dependent on G-protein activation but independent of PLC activation (Canepari and Ogden, 2003). In these experiments, the authors found that sEPSC activation depended upon tyrosine phosphatase activity. In our experiments, blocking tyrosine phosphatase activity with either $1 \mathrm{mM} \mathrm{Na}{ }_{3} \mathrm{VO}_{4}$ or its more potent analog, $100 \mu \mathrm{MbpV}$ (phen), caused a significant ( $\mathrm{p}<0.02$ ) attenuation of T-type current potentiation by DHPG (Fig. 3.5E,F). Interestingly, the potentiation of Ttype currents by mGluR1 also depended on intracellular $\mathrm{Ca}^{2+}$ concentration $\left[\mathrm{Ca}^{2+}\right]_{\text {, }}$, because buffering $\left[\mathrm{Ca}^{2+}\right]_{i}$ with 20 mM intracellular BAPTA in the recording pipette significantly ( $\mathrm{p}<0.02$ ) reduced the effect (Fig. 3.5D,F). To test whether the $\mathrm{Ca}^{2+}$-permeable sEPSC was directly involved in the potentiation of T-type currents by mGluR1, antagonists of this current ( $250 \mu \mathrm{M}$ IEM 1460 or $100 \mu \mathrm{M}$ NA-spermine; (Canepari et al., 2004)) were pre- and co-applied with DHPG and found to have no significant effect on the potentiation magnitude (Fig. 3.5F). Thus, it appears that mGluR1 activates a G-protein and tyrosine-phosphatase-dependent pathway upstream of PLC activation that positively modulates both sEPSC and T-type currents independently of each other.

### 3.2.6 mGluR1 potentiates T-type calcium transients at synaptic sites

mGluR1 receptors and the intracellular pathway leading to the sEPSC are principally localized at the PF-PC synapse in dendritic spines. We used two photon microscopy $\mathrm{Ca}^{2+}$ imaging to determine the localization of the DHPG potentiation effect. Experiments were performed on P9 to P12 rats with heparin $(4 \mathrm{mg} / \mathrm{ml})$ included in the patch pipette in order to block the contaminating effect of $\left[\mathrm{Ca}^{2+}\right]_{\mathrm{i}}$ increase through the mGluR1-activated $\mathrm{IP}_{3} \mathrm{R}$ pathway. Pharmacological antagonists were also used to specifically isolate T-type $\mathrm{Ca}^{2+}$ transients during the whole-cell voltage clamp recordings (see section 3.4.6). In the example shown in Figure 3.6, 40 points-of-interest (POIs) were imaged at a frame rate close to 1 kHz (Fig. 3.6A). As previously shown (Isope and Murphy, 2005), T-type channel mediated $\mathrm{Ca}^{2+}$ transients were observed throughout the PC dendritic tree and the soma (Fig. 3.6).


Figure 3.5 - T-type calcium currents are potentiated by mGluR1 through a signaling pathway that involves G-proteins, intracellular calcium, and tyrosine phosphatases.
A) Control normalized time course showing the effects of DHPG application on voltage-clamped rat PC T-type currents in the absence of any other antagonists (as shown in Figure 3.2C). B) DHPG stimulates T-type currents specifically through mGluR1 receptors. Blocking mGluR1a with perfusion of $100 \mu \mathrm{M}$ LY $367385(\mathrm{n}=8)$ for 20 minutes before and during DHPG application abolished potentiation. C) Potentiation of T-type currents via mGluR1 requires G-protein activation. Substitution of 2 mM GDP- $\beta$-S ( $\mathrm{n}=5$ ) for GTP in the intracellular pipette solution for 10 minutes in the whole-cell conformation eliminated DHPG-induced potentiation. D) The T-type potentiation pathway involves $\mathrm{Ca}^{2+}$-dependent processes. Buffering intracellular $\mathrm{Ca}^{2+}$ through the inclusion of 20 mM BAPTA $(\mathrm{n}=6)$ in the internal solution (whole-cell for $5+$ minutes) attenuated the DHPG-induced potentiation.
E) Potentiation of T-type currents by DHPG requires tyrosine phosphatase activity. Blocking tyrosine phosphatases via perfusion of $100 \mu \mathrm{MbpV}$ (phen) ( $\mathrm{n}=6$ ) for 10 minutes before and during DHPG application attenuated the potentiation effect. F) Histogram showing potentiation values compared to the control (DHPG) potentiation value for the above results as well as for other antagonists. Blocking group
I mGluR receptors with $500 \mu \mathrm{M} \mathrm{MCPG}(\mathrm{n}=6)$ and blocking tyrosine phosphatases with $1 \mathrm{mM} \mathrm{Na}{ }_{3} \mathrm{VO}_{4}$ $(\mathrm{n}=5)$ also significantly ( $\mathrm{p}<0.02$ ) reduced the DHPG-induced increase. Blocking PLC with $1 \mu \mathrm{M}$ U73122 ( $\mathrm{n}=6$ ) or $10 \mu \mathrm{M}$ edelfosine ( $\mathrm{n}=7$ ), serine/threonine kinases (such as PKC) with 1 to $2.5 \mu \mathrm{M}$ staurosporine ( $\mathrm{n}=8$ ), IP ${ }_{3}$ Rs with $1 \mu \mathrm{M}$ xestospongin $\mathrm{C}(\mathrm{n}=6$ ), and sEPSC currents with $250 \mu \mathrm{M}$ IEM $1460(\mathrm{n}=6)$ or $100 \mu$ M NA-spermine ( $\mathrm{n}=5$ ) all caused no significant ( $\mathrm{p}>0.05$ ) change in the level of DHPG-mediated increase in T-type currents. All potentiation values were calculated 100 to 120 seconds after initiation of DHPG application, except for NA-spermine and edelfosine, where the effect was calculated 60 to 80 seconds into potentiation (equivalent time from start of potentiation to other groups), because the effect was delayed. $*$ indicates significance at $\mathrm{p}<0.02$.


Figure 3.6 - DHPG mediates an increase in T-type calcium transients in PCs.
A) Two-photon image of a patch-clamped rat cerebellar PC. The dendritic regions outlined by circles are sites of imaging. Scale bar $=10 \mu \mathrm{M} . \mathbf{B})$ DHPG causes an increase in voltage-clamped low-threshold $\mathrm{Ca}^{2+}$ transients in spines and proximal dendrites when $\mathrm{IP}_{3}$ Rs are blocked. The $\mathrm{Ca}^{2+}$ transients ( $\Delta F /$ Red, see Methods) at individual POIs during depolarizing steps to -45 mV at the soma are shown before (black) and after (red) application of $20 \mu \mathrm{M}$ DHPG. Numbers refer to labels from A. Traces were smoothed. The dotted vertical line indicates onset of the depolarizing pulse. Recordings were performed in the presence of $4 \mathrm{mg} / \mathrm{mL}$ heparin to block $\mathrm{IP}_{3}$ Rs. C) The increase in low threshold $\mathrm{Ca}^{2+}$ transients by mGluR1 activation coincides with the potentiation of T-type somatic currents. Three successive depolarizing pulses at the onset of the DHPG effect are represented, starting 2 min . after the beginning of the application, with 1 min . between pulses. Average $\mathrm{Ca}^{2+}$ transients in all the POIs in the PC are shown in red. The red dotted line represents the standardized baseline $\mathrm{Ca}^{2+}$ level before the pulse; the red solid line identifies the peak of fluorescence before the DHPG effect. In black, current recorded at the soma. The black dotted line represents current baseline while the black solid line identifies the peak current before the DHPG effect. D) Upper panel, the average $\mathrm{Ca}^{2+}$ transient in all imaged spines during the control period is shown in black ( 10 min .) while the average $\mathrm{Ca}^{2+}$ transient after onset of the DHPG effect is shown in red ( 5 min ). Middle panel, Mean current in control period (black) and during DHPG application (red). Lower panel, holding potentials. All data shown above in this figure are from the same cell. E) Left: DHPG causes a potentiation of voltage-clamped T-type $\mathrm{Ca}^{2+}$ transients in the spines, dendrites, and proximal dendrites of PCs when $\mathrm{IP}_{3}$ Rs are not blocked. Proximal dendrites, $\mathrm{n}=20 / 5$ cells, dendrites, $\mathrm{n}=40 / 5$ cells, spines, $\mathrm{n}=55 / 5$ cells, soma, $\mathrm{n}=6 / 5$ cells. ${ }^{* *} \mathrm{p}<0.01$, ${ }^{* * * *} \mathrm{p}<0.001$, (Wilcoxon rank test). Right: DHPG causes a potentiation of T-type $\mathrm{Ca}^{2+}$ transients in the spines and proximal dendrites of PCs when $\mathrm{IP}_{3}$ Rs are blocked with heparin inclusion $(4 \mathrm{mg} / \mathrm{ml})$ in the patch pipette. Variation of the peak $\mathrm{Ca}^{2+}$ transient ( $\Delta \mathrm{F} / \mathrm{red}$ ) normalized to the density of current at the soma (under control conditions; see section 3.4.6) in different compartments of the cell during the control period (black bars) and DHPG application (white bars). Proximal dendrites, $n=33 / 5$ cells, dendrites, $n=52 / 5$ cells, spines, $n=84 / 5$ cells, soma, $n=9 / 5$ cells.

Strikingly, during DHPG $(20 \mu \mathrm{M})$ application, the increase in T-type current recorded at the soma was correlated with: 1) an increase in fluorescence transients in spines but not systematically in their parent dendrites 2) an increase in fluorescence transients in proximal dendrites, and 3) no observed increase in $\mathrm{Ca}^{2+}$ entry in the soma (Fig. 3.6B,C,E). Figure 3.6D shows the average $\mathrm{Ca}^{2+}$ transient from all spines identified in Figure 3.6A both in the control period and during DHPG application. These findings suggest that the effect is localized in the vicinity of mGluR1 receptors, in spines and proximal dendrites. Since the size of the voxel of the 2-photon imaging is greater than the volume of the spine, spine measurements are contaminated by signal coming from the parent dendrite. Because this signal is not potentiated by DHPG, the increase in fluorescence transients observed in spines is underestimated. This most likely explains the larger effect of DHPG on the T-type current than on the fluorescence transient. In order to quantitatively assess the localization of the DHPG effect, the PC was divided into four compartments: soma, proximal dendrites, dendrites and spines. All the POIs for all the cells recorded were then pooled by compartment, normalized by the density of the current in each cell, and averaged together. Histograms in Figure 3.6E show that the $\mathrm{Ca}^{2+}$ transients are larger in spines and that the DHPG effect is significant only in spines ( $\mathrm{p}<0.001$, wilcoxon rank test) and proximal dendrites ( $\mathrm{p}<0.01$, wilcoxon rank test). We also tested the influence of $\mathrm{IP}_{3}$-mediated release of $\mathrm{Ca}^{2+}$ from internal stores by performing the imaging experiments with no heparin in the pipette. When the $\mathrm{IP}_{3} \mathrm{R}$ component was not blocked, DHPG also increased the $\mathrm{Ca}^{2+}$ transients in the shaft of the spiny branchlets (Fig. 3.6E), suggesting that $\mathrm{Ca}^{2+}$ released from internal stores can relay the effect of spine mGluR1 receptor activation to the dendritic shafts.

### 3.2.7 Parallel fiber inputs trigger T-type calcium transients in spines that are potentiated by mGluR1 activation

We have shown that T-type $\mathrm{Ca}^{2+}$ transients and currents evoked by step depolarizations at synaptic sites in PC dendrites are modulated by bath and "puff" applications of DHPG. We then assessed the physiological relevance of this modulation during trains of PF stimulation. High frequency trains, a physiological mode of GC discharge (Chadderton et al., 2004; Jorntell and Ekerot, 2006), are known to be necessary to activate mGluR1 (Takechi et al., 1998). The corresponding EPSPs should be large enough to activate T -type $\mathrm{Ca}^{2+}$ channels (Roth and Hausser, 2001). Experiments were carried out in older wt or Cav 3.1 mice (P16 to P30 mice) using a $\mathrm{K}^{+}$-based internal solution and a near physiological temperature $\left(32^{\circ} \mathrm{C}\right)$. We combined trains of PF stimulation at 100 Hz every 30 sec with PC current clamp recordings (at a holding potential of -75 mV ) and two-photon $\mathrm{Ca}^{2+}$ imaging. Heparin $(4 \mathrm{mg} / \mathrm{ml})$ was included in the patch pipette and cyclopiazonic acid (CPA, $6 \mu \mathrm{M}$ ) in the bath to ensure a complete block of internal $\mathrm{Ca}^{2+}$ stores, as mGluR1 also increases intracellular $\mathrm{Ca}^{2+}$ levels through a welldocumented activation of $\mathrm{IP}_{3}$ Rs (Finch and Augustine, 1998; Takechi et al., 1998) . AM 251, a
cannabinoid antagonist, was also perfused to prevent presynaptic depression of the PF input (Crepel and Daniel, 2007; Maejima et al., 2005). In wt animals, all cells tested displayed a small and graded local $\mathrm{Ca}^{2+}$ transient in PC spines and spiny branchlets (Fig. 3.7A,B). This signal became detectable after the third or the fourth stimulation in the 100 Hz train (Fig. 3.7C; n=6) and increased after each of the following stimulations. In all of the recordings, the size of the first EPSP in the train was used to monitor the strength of the stimulation (mean unitary EPSP size: $2.2 \mathrm{mV}, \mathrm{n}=6$; Fig. 3.7). It should be noted that regenerative $\mathrm{Ca}^{2+}$ spikes were never induced in the dendrites when stimulating the PFs only. Using the $\mathrm{Ca}_{\mathrm{v}} 3.1 \mathrm{KO}$ mice, we show that a major component of the local $\mathrm{Ca}^{2+}$ transients evoked by a train of PF stimulation in target spines is mediated by Cav 3.1 T-type $\mathrm{Ca}^{2+}$ channels, since the mean $\Delta \mathrm{F} / \mathrm{R}$ was $4.67 \pm 1.2 \%(n=6)$ in wt mice and $1 \pm 0.3 \%$ in KO mice ( $n=6$; Fig. 3.7D). In wt mice, perfusion of JNJ16259685 ( $1.5 \mu \mathrm{M}$ ), a very potent and specific antagonist of the mGluR1 receptor (Knopfel, 2007), strongly reduced local $\mathrm{Ca}^{2+}$ transients (mean $\Delta \mathrm{F} / \mathrm{R}=2.45 \pm 0.9 \%$; Fig. 3.7C,D), suggesting that the train of PF stimulation underlies an mGluR1 receptor-mediated potentiation of the local $\mathrm{Ca}^{2+}$ transient. This effect occurred with a fast time course (Fig. 3.7C) that is consistent with the penetration kinetics of the antagonist in the slice. Moreover, in $\mathrm{Ca}_{\mathrm{v}} 3.1 \mathrm{KO}$ mice, no effect was observed after application of JNJ16259685 ( $1.5 \mu \mathrm{M}$ ) (mean $\Delta \mathrm{F} / \mathrm{R}=1.2 \pm 0.28 \%$; Fig. 3.7D). Thus, bursts of PF EPSPs can activate $\mathrm{Ca}_{\mathrm{v}} 3.1-$ mediated $\mathrm{Ca}^{2+}$ transients that are strongly potentiated by mGluR1 activation, and therefore, activation of the mGluR1 receptor by trains of PFs can locally and selectively modulate $\mathrm{Ca}^{2+}$ signaling in PC dendrites.


Figure 3.7 - A burst of PF stimulation modulates $\mathrm{Ca}_{\mathbf{v}} 3.1$ calcium channels via mGluR1 receptors.
A) Schematic drawing of experimental arrangement. The stimulation pipette is in blue while the recording pipette is in black, with an inset representative EPSP recorded at the soma following PF stimulation. B) Fluorescence transients ( $\Delta \mathrm{F} / \mathrm{R}$ ) in spiny branchlets of the mouse PC positioned on a contrast enhanced 2-photon section. In red, average fluorescence of 3-7 POIs in the regions of interest outlined with white dashed-lines. Note the local and graded $\mathrm{Ca}^{2+}$ transients. In blue, position of the stimulations delivered in PFs (11 stimulations/ 100 Hz ). C) Left panel, reduction (red dashed line) of the PF-induced $\mathrm{Ca}^{2+}$ transient following perfusion of JNJ16259685 ( $1.5 \mu \mathrm{M}$ ). Each trace is an average of 5 consecutives trials. Fluorescence traces are an averaging of all responsive spiny branchlets. Right panel, time course of the size the first EPSP in the train (black) and change of $\mathrm{Ca}^{2+}$ fluorescence (red) following
perfusion of JNJ16259685 ( $1.5 \mu \mathrm{M}$ ) in all of the cells tested (n=6). D) Effect of JNJ16259685
application on the change of $\mathrm{Ca}^{2+}$ fluorescence in $\mathrm{wt}(\mathrm{n}=6)$ and $\mathrm{Ca}_{\mathrm{v}} 3.1-/$ - mice $(\mathrm{n}=6)$. Left panel, individual cells, Right panel, average histograms. *p $<0.05$ Wilcoxon Matched-Pairs Signed-Ranks Test. **p<0.01 Wilcoxon test.

### 3.3 Discussion

### 3.3.1 mGluR1 potentiation of Cav 3.1 T-type calcium channels

We report here that recombinant and native $\mathrm{Ca}_{\mathrm{v}} 3.1 \mathrm{~T}$-type $\mathrm{Ca}^{2+}$ currents are potentiated by mGluR1 activation. In recombinant expression systems mGluR1 modulation is shown to be subtypespecific, with a potentiation of $\mathrm{Ca}_{\mathrm{v}} 3.1$ and $\mathrm{Ca}_{\mathrm{v}} 3.2$ isoforms and an inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.3$ channels. The latter is consistent with the known inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.3$ channels by $\mathrm{G}_{\mathrm{q} / 11}$-coupled mAChRs (Hildebrand et al., 2007).

In PCs from young rodents (P7-P15) T-type currents can be isolated and are mainly mediated by the $\mathrm{Ca}_{\mathrm{v}} 3.1$ channels. As previously reported (Isope and Murphy, 2005), young PC T-type currents have a low sensitivity to $\mathrm{Ni}^{2+}$, indicating little functional expression of $\mathrm{Ca}_{\mathrm{v}} 3.2$, and their biophysical properties match closer with $\mathrm{Ca}_{\mathrm{v}} 3.1$ rather than $\mathrm{Ca}_{\mathrm{v}} 3.3$ currents. We show here that T -type $\mathrm{Ca}^{2+}$ currents are undetectable in PCs from young $\mathrm{Ca}_{\mathrm{v}} 3.1 \mathrm{KO}$ mice. These findings are in agreement with in situ hybridization on 250-350 g Sprague Dawley rats that revealed very high expression of $\mathrm{Ca}_{\mathrm{v}} 3.1$ in PCs, with $\mathrm{Ca}_{\mathrm{v}} 3.2$ and $\mathrm{Ca}_{\mathrm{v}} 3.3$ signals below detection levels (Talley et al., 1999). However, an immunohistochemistry study showed high expression of Cav 3.3 in PCs of P14 to P21 Sprague Dawley rats (Molineux et al., 2006) suggesting that $\mathrm{Ca}_{\mathrm{v}} 3.3$ expression may increase during the third week of cerebellar development. Indeed, in our imaging experiments in 3-4 week old Cav 3.1 KO mice, a small component of the PF-induced subthreshold $\mathrm{Ca}^{2+}$ transient remained that may be mediated by $\mathrm{Ca}_{\mathrm{v}} 3.3$ (Fig. 3.7D).

When DHPG, an agonist of mGluR1 receptors, was applied onto acute cerebellar slices from young rats or mice, PC T-type $\mathrm{Ca}^{2+}$ currents were robustly and reversibly potentiated as expected if $\mathrm{Ca}_{\mathrm{v}} 3.1$ subunits, but not $\mathrm{Ca}_{\mathrm{v}} 3.3$ subunits, predominate. As observed in the heterologous system expressing $\mathrm{Ca}_{\mathrm{v}} 3.1$, mGluR1-mediated potentiation did not alter the activation or inactivation kinetics of the T-type current. Using fast optical mapping of $\mathrm{Ca}^{2+}$ influx we show that the T-type mediated $\mathrm{Ca}^{2+}$ influx is widespread in the PC soma and dendrites. However, the mGluR1-mediated potentiation is restricted to the spines and proximal dendrites, where these metabotropic receptors are found in association with PF and CF synapses, respectively (Lopez-Bendito et al., 2001).

### 3.3.2 Signal transduction pathway

As shown in Figure 3.1, activation of mGluR1a with glutamate in the HEK expression system causes a potentiation of $\mathrm{Ca}_{\mathrm{v}} 3.1$ and $\mathrm{Ca}_{\mathrm{v}} 3.2$ currents with a slow and delayed onset. In contrast, mGluR1a activation causes a rapid and saturating inhibition of the $\mathrm{Ca}_{\mathrm{v}} 3.3$ currents similar to that shown for the muscarinic modulation of Cav 3.3 T-type currents in the same system (Hildebrand et al., 2007). Since the same $\mathrm{Ga}_{q / 11}$ protein is coupled to the metabotropic receptors that cause these modulations of $\mathrm{Ca}_{\mathrm{v}} 3.1$,
$\mathrm{Ca}_{\mathrm{v}} 3.2$, and $\mathrm{Ca}_{\mathrm{v}} 3.3$ currents, we postulate that different downstream transduction pathways in combination with specific channel isoforms determines the sign of the T-type modulation (see also (Iftinca et al., 2007)). Indeed, we have previously shown that the intracellular pathway leading to the inhibition of the $\mathrm{Ca}_{\mathrm{v}} 3.3$ currents is independent from classical $\mathrm{PLC} / \mathrm{IP}_{3} \mathrm{R}$ pathways, $\mathrm{Ca}^{2+}$-independent, but partially G $\beta \gamma$-dependant. In contrast to this "inhibition" pathway, the mGluR1-mediated "potentiation" of $\mathrm{Ca}_{v} 3.1$ currents within PCs is also $\mathrm{PLC} / \mathrm{IP}_{3} \mathrm{R} / \mathrm{Ca}^{2+}$ store-independent, but depends on tyrosine phosphatase activity and intracellular $\mathrm{Ca}^{2+}$ signals, as blocking tyrosine phosphatases or buffering $\mathrm{Ca}^{2+}$ using high concentration of BAPTA attenuates the T-type current stimulation. Although non-classical, this PLC-independent, tyrosine phosphatase-dependent pathway was already described at the PF-PC synapse by Canepari and Ogden (2003) between the mGluR1 receptor and the sEPSC, identified as the non-specific cationic channel TRPC1 (Kim et al., 2003), and also between mGluR1 and TRP channels in hippocampal inhibitory interneurons (Topolnik et al., 2006). Canepari and Ogden showed that photolysis of glutamate in cerebellar slices activates the sEPSC via a G protein and protein tyrosine kinase/phosphatase pathway. Protein tyrosine kinase (PTK) inhibitor enhanced the sEPSC while protein tyrosine phosphatase inhibitor blocked this current. We similarly observed a nonsignificant potentiation of the T-type current using $2.5 \mu \mathrm{M}$ staurosporine, a broad antagonist of protein kinases like PKC and PTK.

Since sEPSC antagonists (IEM 1460 or NA-spermine) have no effect on the T-type current modulation, the sEPSC channels and T-type channels appear to be independently regulated by mGluR1 activity. Blocking $\mathrm{IP}_{3}$ Rs also does not alter the mGluR1-mediated potentiation of T-type currents, leading to the hypothesis that the intracellular $\mathrm{Ca}^{2+}$ signal required for this T-type potentiation could be due to $\mathrm{Ca}^{2+}$ influx through the T-type channel itself, with possible contributions from either the sEPSC channels or $\mathrm{IP}_{3}$ Rs not being ruled out. In support of this notion, it was observed that the mGluR1mediated potentiation of T-type $\mathrm{Ca}^{2+}$ influx spread further along the dendritic shaft from the putative mGluR1 synaptic PF and CF microdomains when $\mathrm{IP}_{3}$ Rs were not blocked with heparin, consistent with past literature on mGluR1-induced, $\mathrm{IP}_{3}$ R-dependent $\mathrm{Ca}^{2+}$ waves (Nakamura et al., 1999).

### 3.3.3 Physiological implications of alterations in T-type biophysical properties

Our data indicate that the mGluR1-dependent modulation of $\mathrm{Ca}_{\mathrm{v}} 3.1$ channels involves a modification of channel gating properties rather than a change in channel expression; the effect can be induced within seconds by agonist application and the potentiation involves a shift in the activation curve (Fig. 3.3). In support of a direct T-type channel interaction, tyrosine phosphatase activity has been shown to facilitate $\mathrm{Ca}_{\mathrm{v}} 3$ currents through a proposed transition to a high conductance state in spermatogenic cells (Arnoult et al., 1997). The observed alteration of T-type biophysical properties by mGluR1a activation has potential physiological implications. Interestingly, the mGluR1a-induced shift in
the activation curve of the $\mathrm{Ca}_{\mathrm{v}} 3.1$ channels leads to a more efficient potentiation at potentials between -45 mV and -40 mV . At -45 mV , the T-type current increased two-fold upon mGluR1 activation while only $20 \%$ at -20 mV . The stringent recording conditions used for the characterization of these biophysical parameters (see sections 3.2 and 3.4) make it unlikely that poor voltage-clamp may account for the activation shift. We show here that this activation shift can significantly influence dendritic excitability and synaptic integration. mGluR1 activation decreases the threshold for T-type $\mathrm{Ca}^{2+}$ spike generation (Fig. 3.4) when other conductances are blocked and reduces the latency of the first spike appearance upon depolarization in physiological conditions. Parallel fiber and/or CF activation of mGluR1 receptors and subsequent potentiation of T-type $\mathrm{Ca}^{2+}$ conductances may thus promote PC transitions from the hyperpolarized state to the depolarized state, in which synaptic integration and spike coding can proceed (Loewenstein et al., 2005).

### 3.3.4 mGluR1 potentiation of $\mathbf{C a}_{\mathbf{v}}$ 3.1-mediated calcium influx in response to synaptic activity

We imaged $\mathrm{Ca}^{2+}$ influx in response to trains of PF stimulations and found that the response was local and significantly smaller in $\mathrm{Ca}_{\mathrm{v}} 3.1 \mathrm{KO}$ mice compared to control mice. Interestingly, the first EPSP in the train did not induce any $\mathrm{Ca}^{2+}$ influx, suggesting that temporal summation is required to reach the threshold for T-type channel activation. Based on pairs of GC and PC recordings (Isope and Barbour, 2002), we calculate that about 30 PF inputs (mean unitary connection: 8.4 pA ; conversion factor: $8.3 \mu \mathrm{~V} / \mathrm{pA}$ ) are required to generate an EPSP of 2 mV at the soma (mean unitary value for the results in Fig. 3.7). Roth and Hausser (2001) showed that an EPSP recorded at the soma is attenuated by 6 -fold compared to synaptic locations, indicating that a 2 mV EPSP at the soma corresponds to a 12 mV depolarization at synaptic sites. Because hyperpolarized potentials are necessary to remove the inactivation of T-type currents, 12 mV is indeed too small to span the gap to the foot of the T-type activation curve. High frequency bursts around 100 Hz may produce sufficient temporal summation to lead to T-type channel opening. In addition, recent results suggest the existence of a membrane potential overshoot in the spine head when compared to the parent shaft due to the neck resistance (Araya et al., 2006). This electrical amplification may be regulated, as assessed by measuring the spine-dendrite diffusion coefficient (Bloodgood and Sabatini, 2005; Svoboda et al., 1996) and could permit local T-type channel activation. By shifting the activation curve and potentiating T-type conductance at spines receiving a burst of EPSPs, mGluR1 activation could efficiently enhance the $\mathrm{Ca}^{2+}$ influx at the active spine relative to nearby inactive spines (see Figs. 3.6 and 3.7). As suggested recently for sodium channels (Araya et al., 2007), $\mathrm{Ca}^{2+}$ currents could in turn boost spine depolarization, potentially affecting the transmission of the EPSPs to the soma. Overall, our results unravel a novel mechanism generating
spine-specific, frequency-dependent $\mathrm{Ca}^{2+}$ signaling that might play an important role in dendritic integration and plasticity.

### 3.4 Experimental procedures

### 3.4.1 HEK 293 cell culture, transfection, and electrophysiology

All equipment, procedures, and reagents were used as described in detail for the study of modulation of recombinant T-type channels by mAChRs (Hildebrand et al., 2007). Briefly, HEK 293 cells that stably expressed either rat $\mathrm{Ca}_{\mathrm{v}} 3.1, \mathrm{Ca}_{\mathrm{v}} 3.2$ or $\mathrm{Ca}_{\mathrm{v}} 3.3$ subunits were transiently transfected with mGluR1a using Lipofectamine (Invitrogen) and perforated patch recordings (with $\beta$-Escin) were performed 48 hours later. The external recording solution contained (in mM): $2 \mathrm{CaCl}_{2}, 1 \mathrm{MgCl}_{2}, 10$ HEPES, $40 \mathrm{TEACl}, 92 \mathrm{CsCl}, 10$ glucose, $\mathrm{pH}=7.4$, while the internal pipette solution contained (in mM ): 120 CsMethanesulfonate, 11 EGTA, 10 HEPES, $2 \mathrm{MgCl}_{2}$, and $75-100 \mu \mathrm{M} \beta$-Escin (for perforated patch) $\mathrm{pH}=7.2$. During pharmacology experiments, cells were considered "potentiated" or "inhibited" when they displayed $\mathrm{a}>10 \%$ modulating effect with a clear exponential time course. All remaining cells were grouped into a "no effect" group. To quantify the effect of $100 \mu \mathrm{M}$ glutamate, peak current levels were allowed to reach equilibrium and then 2 to 5 values were averaged.

### 3.4.2 Animals

All experimental procedures involving animals and their care were performed in accordance with recommendations of the Canadian Council on Animal Care and the regulations and policies of the University of British Columbia Animal Care Facility and the University Animal Care Committee. Mice lacking the cacnale gene (encoding $\mathrm{Ca}_{\mathrm{v}} 2.3$ ) and the cacnalg were respectively produced as previously described (Pereverzev et al., 2002) (Petrenko et al., 2007). All animals were bred under an identical C57/B16 background, and litter mate controls were used where possible.

### 3.4.3 Slice preparation

Male Wistar rats (8-12 days old, but mostly 9-10 days old) or male CBL57/B16 mice or mutant mice ( $8-12$ days old) were anaesthetized with halothane and decapitated. The head was immediately chilled over ice and the cerebellar vermis was removed with a scalpel and placed in ice cold bicarbonatebuffered saline (BBS) solution containing (in mM): $120 \mathrm{NaCl}, 3 \mathrm{KCl}, 26 \mathrm{NaHCO}_{3}, 1.25 \mathrm{NaH}_{2} \mathrm{PO}_{4}, 2$ $\mathrm{CaCl}_{2}, 1 \mathrm{MgCl}_{2}$, 20 glucose, 1 kynurenate, 0.1 picrotoxin. During cutting, incubating, and recording, slices were constantly bubbled with $95 \% \mathrm{O}_{2} / 5 \% \mathrm{CO}_{2}$ (carbogen). The vermis was glued in the sagittal orientation to the stage of a Vibratome 1500 Sectioning System (MO, USA) and 200 to $250 \mu \mathrm{~m}$ sagittal
slices were cut from the cerebellar vermis, transferred to BBS at $32^{\circ} \mathrm{C}$, and allowed to cool down passively to room temperature.

For imaging and KO mice experiments, slices were cut in a protecting solution containing (in mM ): 130 KGluconate, $14.6 \mathrm{KCl}, 2$ EGTA, 20 HEPES, 25 Glucose, D-APV 0.05 and 0.00005 minocycline. Before the transfer into normal BBS solution, slices were soaked in sucrose-based solution containing (in mM): 230 sucrose, $2.5 \mathrm{KCl}, 26 \mathrm{NaHCO} 3,1.25 \mathrm{NaHPO} 4,25$ Glucose, $0.8 \mathrm{CaCl} 2,8$ MgCl 2 , D-APV 0.05 and 0.00005 minocycline. For PF stimulation experiments older animals (16-30 days old) were used. All experiments were performed on Wistar rats, except for the experiments shown in Figure 3.2E,F and Figure 3.7, where wt, $\mathrm{Ca}_{\mathrm{v}} 2.3$, and $\mathrm{Ca}_{\mathrm{v}} 3.1 \mathrm{KO}$ mice were used as indicated.

### 3.4.4 Electrophysiological recordings

Slices were transferred to a Warner RC-26G recording chamber (total working volume $=\sim 250$ $\mu \mathrm{L}$; CT, USA) and perfused with bubbling modified BBS external solution, containing (in mM): 120 $\mathrm{NaCl}, 3 \mathrm{KCl}, 26 \mathrm{NaHCO}_{3}, 1.25 \mathrm{NaH}_{2} \mathrm{PO}_{4}, 2 \mathrm{CaCl}_{2}, 1 \mathrm{MgCl}_{2}, 20$ glucose, 1 kynurenate, 0.1 picrotoxin, 0.3 tetrodotoxin, $5 \mathrm{TEACl}, 14$-aminopyridine, $0.05 \mathrm{NiCl}_{2}$, and $0.02 \mathrm{CdCl}_{2}$. The $20 \mu \mathrm{M} \mathrm{Cd}^{2+}$ was used as an effective antagonist of all HVA $\mathrm{Ca}^{2+}$ currents that left T-type currents unaffected (Tai et al., 2006), while $50 \mu \mathrm{M} \mathrm{Ni}^{2+}$ blocked R-type but not $\mathrm{Ca}_{\mathrm{v}} 3.1$ or Cav 3.3 currents (Lee et al., 1999; Zamponi et al., 1996). Cerebellar PCs were visually identified using a Zeiss Axioskop 2 microscope with an Achroplan 60 X water immersion lens. Whole-cell patch clamp recordings from PCs were performed using a Multiclamp 700B amplifier and Digidata 1322A (MDS Analytical Technologies), controlled and monitored with a computer running pClamp9 software (MDS Analytical Technologies). Patch pipettes (borosilicate glass BF150-86-10; Sutter Instruments; CA, USA), were pulled using a Sutter P-87 puller and had typical resistances of 3-5 $\mathrm{M} \Omega$ when filled with internal solution.

For voltage-clamp experiments, the internal solution contained (in mM): 140 CsMethanesulfonate, $5 \mathrm{TEACl}, 0.5 \mathrm{MgCl}_{2}, 10$ HEPES, $4 \mathrm{MgATP}, 0.5 \mathrm{Na}_{3} \mathrm{GTP}$, and 0.3 EGTA, adjusted to $\mathrm{pH}=7.3, \sim 290 \mathrm{mOsm}$. In specific pharmacology experiments, 0.5 mM Na 3 GTP was replaced with 2 mM GDP- $\beta$-S. Cells were held at a holding potential of $\mathrm{V}_{\mathrm{h}}=-75 \mathrm{mV}$. Cells with leak current above 600 pA at $\mathrm{V}_{\mathrm{h}}=-75 \mathrm{mV}$ were discarded. Data were low-pass filtered at 2 kHz using the built-in Bessel filter of the amplifier, with sampling at 20 kHz . Series resistance was compensated between 70 and $80 \%$ on every cell. Leak subtraction of capacitance current and $\mathrm{I}_{\mathrm{L}}$ was performed on-line using a $\mathrm{P} / 6$ protocol with reverse polarity pulses for channel kinetics experiments and with Clampfit9 (MDS Analytical Technologies) during offline analysis for all other experiments. Both the leak-subtracted and non leaksubtracted traces were acquired through separate channels for all protocols. Current-voltage
relationships and channel activation, inactivation, and deactivation rates were analyzed as previously described (Hildebrand et al., 2007).

For current clamp experiments, the external solution (termed ACSF) contained (in mM ): 125 $\mathrm{NaCl}, 3 \mathrm{KCl}, 26 \mathrm{NaHCO}_{3}, 1.25 \mathrm{NaH}_{2} \mathrm{PO}_{4}, 2 \mathrm{CaCl}_{2}, 2 \mathrm{MgCl}_{2}, 10$ glucose, 1 kynurenate and 0.1 picrotoxin and the internal solution contained (in mM): 150 KGluconate, $4 \mathrm{NaCl}, 10$ HEPES, 10 MgATP and $0.5 \mathrm{Na}_{3}$ GTP, adjusted to $\mathrm{pH}=7.3$ and 295 mOsm . For T-type spike experiments, the voltage-clamp internal solution was used. Recordings were low-pass filtered at 10 kHz , with sampling at 50 kHz . Input resistance was calculated and averaged from the voltages measured at equilibrium during small depolarizing and hyperpolarizing currents (between -50 pA and +50 pA ) that elicited no dramatic voltage-gated changes (see Fig. 3.4A for example). To determine the inflection point where T-type spikes or APs are initiated, the slopes of the relevant traces were extrapolated and plotted in Microcal Origin (Version 7.5, Northampton, MA) and the inflection point where the slope stops decreasing and starts increasing was determined. The time value at this point represents the spike latency and the corresponding membrane voltage value from the original trace represents the V threshold. The maximum slope $\left(\mathrm{dV} / \mathrm{dt}_{\max }\right)$ was also determined from these differentiation plots.

All recordings were performed at room temperature $\left(20-24^{\circ} \mathrm{C}\right)$ except for PF stimulation where slices were maintained at $32^{\circ} \mathrm{C}$. Figures and fittings utilized the software program Microcal Origin. Statistical significance was determined by Student's T-Tests or non parametric Wilcoxon rank test, and significant values were set as indicated in the text and figure legends.

### 3.4.5 Compounds and perfusion

(S)-3,5-DHPG, LY367385, (S)-MCPG, U73122, IEM 1460, and SKF 96365 were all obtained from Tocris Cookson (MO, USA). Edelfosine and bpV (phen) was obtained from Calbiochem (CA, USA). All other drugs were ordered from Sigma-Aldrich (MO, USA). Drugs were dissolved in $\mathrm{dH}_{2} \mathrm{O}$, equimolar NaOH , or DMSO, according to manufacturers solubility data. The highest concentration of DMSO in the recording solution did not exceed $0.1 \%$, a concentration that did not detectably affect $\mathrm{Ca}^{2+}$ channel properties. Cells were gravity perfused with solutions at flow rates $>1 \mathrm{ml} /$ minute through high chemical-resistant PTFE tubing (Cole-Parmer Instrument Company; Il, USA). A closed perfusion system ( $5-20 \mathrm{ml}$ ) was also used to maintain high concentrations of antagonists and agonists during experiments, with separate lines containing control or DHPG solutions. During washout experiments, the perfusion system was open with solution flowing into a waste container. For a more physiological activation of mGluR1, DHPG was puffed directly onto the recorded PC using a 50 ms , 10 PSI pressure pulse delivered by a Parker Hannifin Picospritzer III to a patch pipette loaded with $100 \mu \mathrm{M}$ DHPG and placed within close proximity of the PC. Control recordings showed that puffing on antagonist was able
to block T-type currents (puffing $100 \mu \mathrm{M}$ SKF 96365 inhibited T-type current by $28 \%+/-4 \%, \mathrm{n}=4$, while puffing control BBS had no effect on T-type currents ( $2 \%+/-1 \%, n=2$ ) ).

### 3.4.6 Two-photon imaging

Calcium transients in PCs were imaged with a custom-built multi-photon laser scanning microscope in which both X and Y scanning are operated by acousto-optic deflectors (AOD; A-A OptoElectronics; based on the AA.DTS.XY-250 model). Two-photon excitation was produced by an infrared Ti-Sa pulsed laser (Tsunami pumped by a Millenia VI, Spectra-Physics) set to 825 nm and tuned to 700 fs to mitigate AOD-induced dispersion. In this non-mechanical scanning microscope, deflection of the laser beam to a specific position is obtained by setting the appropriate acoustic wavelength in the AOD crystal. Switching the illumination between any two points takes $4 \mu$ s permitting random access imaging at high frame rates. National Instrument boards programmed under Labview were used to implement digital scanning strategies and to synchronize AOD scanning and photon-counting detection with a cooled AsGaP photomultiplier (H7421-40, Hamamatsu).

Whole cell recordings were performed with an Axoclamp amplifier (Axon Instruments). PCs were visualized using a combination of gradient contrast and on-line video contrast enhancement (CoolSnapCf and Metamorph, Roper Scientific) at wavelengths of 670-740 nm. Pipettes were filled with intracellular solution that was supplemented with a morphological dye (Alexa 594, 10-20 $\mu \mathrm{M}$, Molecular Probes) and a $\mathrm{Ca}^{2+}$-sensitive dye (Fluo5F $400 \mu \mathrm{M}$ ). In order to block P/Q-type $\mathrm{Ca}^{2+}$ channels, slices were pre-incubated for at least 30 min in a chamber containing 1 ml of BBS supplemented with $1 \mu \mathrm{M}$ Agatoxin IVA/ $0.1 \% \mathrm{BSA}$ (except for the PF stimulation experiment). Besides the addition of Agatoxin IVA and the omission of $\mathrm{Cd}^{2+}$, the same modified BBS external solution was used as for the above whole-cell voltage-clamp recordings. Pipettes resistance ranged from 3 to $5 \mathrm{M} \Omega$. Current was injected to hold the cells at membrane potentials of -60 to -65 mV . PC imaging was started after at least 30 min of whole-cell dialysis. Spines were resolved and POIs were placed on spines' heads and attached dendritic shafts. Optical transients were subsequently monitored simultaneously in 10 to 50 spines and dendrites at frame rates close to 1 kHz (dwell time 20-50 $\mu \mathrm{s}$ ). In this multiunit recording mode, the POI sequence is sampled repetitively and the signal for each location is displayed online as a continuous or episodic recording and analyzed offline using custom routines written in Igor (Wavemetrics). Variation of fluorescence was calculated as a ratio of the change in fluorescence $(\Delta \mathrm{F})$ of the $\mathrm{Ca}^{2+}$ dye over the fluorescence (R) of the red morphological dye (Alexa 594 ), labeled $\Delta F / R$. This strategy overcomes movements and loading artifacts and errors due to changes in basal $\mathrm{Ca}^{2+}$ concentration.

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### 3.6 References

Aiba, A., Kano, M., Chen, C., Stanton, M.E., Fox, G.D., Herrup, K., Zwingman, T.A., and Tonegawa, S. (1994). Deficient cerebellar long-term depression and impaired motor learning in mGluR1 mutant mice. Cell 79, 377-388.

Anderson, M.P., Mochizuki, T., Xie, J., Fischler, W., Manger, J.P., Talley, E.M., Scammell, T.E., and Tonegawa, S. (2005). Thalamic Cav 3.1 T-type $\mathrm{Ca}^{2+}$ channel plays a crucial role in stabilizing sleep. Proc Natl Acad Sci U S A 102, 1743-1748.

Araya, R., Jiang, J., Eisenthal, K.B., and Yuste, R. (2006). The spine neck filters membrane potentials. Proc Natl Acad Sci U S A 103, 17961-17966.

Araya, R., Nikolenko, V., Eisenthal, K.B., and Yuste, R. (2007). Sodium channels amplify spine potentials. Proc Natl Acad Sci U S A 104, 12347-12352.

Arnoult, C., Lemos, J.R., and Florman, H.M. (1997). Voltage-dependent modulation of T-type calcium channels by protein tyrosine phosphorylation. Embo J 16, 1593-1599.

Baude, A., Nusser, Z., Roberts, J.D., Mulvihill, E., McIlhinney, R.A., and Somogyi, P. (1993). The metabotropic glutamate receptor (mGluR1 alpha) is concentrated at perisynaptic membrane of neuronal subpopulations as detected by immunogold reaction. Neuron 11, 771-787.

Bloodgood, B.L., and Sabatini, B.L. (2005). Neuronal activity regulates diffusion across the neck of dendritic spines. Science 310, 866-869.

Bourinet, E., Alloui, A., Monteil, A., Barrere, C., Couette, B., Poirot, O., Pages, A., McRory, J., Snutch, T.P., Eschalier, A., and Nargeot, J. (2005). Silencing of the Cav 3.2 T-type calcium channel gene in sensory neurons demonstrates its major role in nociception. Embo J 24, 315-324.

Canepari, M., Auger, C., and Ogden, D. (2004). $\mathrm{Ca}^{2+}$ ion permeability and single-channel properties of the metabotropic slow EPSC of rat Purkinje neurons. J Neurosci 24, 3563-3573.

Canepari, M., and Ogden, D. (2003). Evidence for protein tyrosine phosphatase, tyrosine kinase, and Gprotein regulation of the parallel fiber metabotropic slow EPSC of rat cerebellar Purkinje neurons. J Neurosci 23, 4066-4071.

Chadderton, P., Margrie, T.W., and Hausser, M. (2004). Integration of quanta in cerebellar granule cells during sensory processing. Nature 428, 856-860.

Chemin, J., Monteil, A., Perez-Reyes, E., Bourinet, E., Nargeot, J., and Lory, P. (2002). Specific contribution of human T-type calcium channel isotypes (alpha ${ }_{1 \mathrm{G}}$, alpha $\mathrm{a}_{1 \mathrm{H}}$ and alpha ${ }_{1 \mathrm{I}}$ ) to neuronal excitability. J Physiol 540, 3-14.

Chen, C.C., Lamping, K.G., Nuno, D.W., Barresi, R., Prouty, S.J., Lavoie, J.L., Cribbs, L.L., England, S.K., Sigmund, C.D., Weiss, R.M., et al. (2003). Abnormal coronary function in mice deficient in alpha $_{1 H}$ T-type $\mathrm{Ca}^{2+}$ channels. Science 302, 1416-1418.

Christie, B.R., Schexnayder, L.K., and Johnston, D. (1997). Contribution of voltage-gated $\mathrm{Ca}^{2+}$ channels to homosynaptic long-term depression in the CA1 region in vitro. J Neurophysiol 77, 1651-1655.

Conquet, F., Bashir, Z.I., Davies, C.H., Daniel, H., Ferraguti, F., Bordi, F., Franz-Bacon, K., Reggiani, A., Matarese, V., Conde, F., and et al. (1994). Motor deficit and impairment of synaptic plasticity in mice lacking mGluR1. Nature 372, 237-243.

Crepel, F., and Daniel, H. (2007). Developmental changes in agonist-induced retrograde signaling at parallel fiber-Purkinje cell synapses: role of calcium-induced calcium release. J Neurophysiol 98, 25502565.

Crunelli, V., Cope, D.W., and Hughes, S.W. (2006). Thalamic T-type $\mathrm{Ca}^{2+}$ channels and NREM sleep. Cell Calcium 40, 175-190.

Egger, V., Svoboda, K., and Mainen, Z.F. (2005). Dendrodendritic synaptic signals in olfactory bulb granule cells: local spine boost and global low-threshold spike. J Neurosci 25, 3521-3530.

Finch, E.A., and Augustine, G.J. (1998). Local calcium signalling by inositol-1,4,5-trisphosphate in Purkinje cell dendrites. Nature 396, 753-756.

Hartmann, J., Blum, R., Kovalchuk, Y., Adelsberger, H., Kuner, R., Durand, G.M., Miyata, M., Kano, M., Offermanns, S., and Konnerth, A. (2004). Distinct roles of Galpha(q) and Galpha11 for Purkinje cell signaling and motor behavior. J Neurosci 24, 5119-5130.

Hildebrand, M.E., David, L.S., Hamid, J., Mulatz, K., Garcia, E., Zamponi, G.W., and Snutch, T.P. (2007). Selective inhibition of Cav3.3 T-type calcium channels by Galphaq/11-coupled muscarinic acetylcholine receptors. J Biol Chem 282, 21043-21055.

Ichise, T., Kano, M., Hashimoto, K., Yanagihara, D., Nakao, K., Shigemoto, R., Katsuki, M., and Aiba, A. (2000). mGluR1 in cerebellar Purkinje cells essential for long-term depression, synapse elimination, and motor coordination. Science 288, 1832-1835.

Iftinca, M., Hamid, J., Chen, L., Varela, D., Tadayonnejad, R., Altier, C., Turner, R.W., and Zamponi, G.W. (2007). Regulation of T-type calcium channels by Rho-associated kinase. Nat Neurosci 10, 854860.

Ikeda, H., Heinke, B., Ruscheweyh, R., and Sandkuhler, J. (2003). Synaptic plasticity in spinal lamina I projection neurons that mediate hyperalgesia. Science 299, 1237-1240.

Isope, P., and Barbour, B. (2002). Properties of unitary granule cell-->Purkinje cell synapses in adult rat cerebellar slices. J Neurosci 22, 9668-9678.

Isope, P., and Murphy, T.H. (2005). Low threshold calcium currents in rat cerebellar Purkinje cell dendritic spines are mediated by T-type calcium channels. J Physiol 562, 257-269.

Joksovic, P.M., Nelson, M.T., Jevtovic-Todorovic, V., Patel, M.K., Perez-Reyes, E., Campbell, K.P., Chen, C.C., and Todorovic, S.M. (2006). CaV3.2 is the major molecular substrate for redox regulation of T-type Ca2+ channels in the rat and mouse thalamus. J Physiol 574, 415-430.

Jorntell, H., and Ekerot, C.F. (2006). Properties of somatosensory synaptic integration in cerebellar granule cells in vivo. J Neurosci 26, 11786-11797.

Kim, D., Song, I., Keum, S., Lee, T., Jeong, M.J., Kim, S.S., McEnery, M.W., and Shin, H.S. (2001). Lack of the burst firing of thalamocortical relay neurons and resistance to absence seizures in mice lacking alpha(1G) T-type $\mathrm{Ca}(2+$ ) channels. Neuron 31, 35-45.

Kim, S.J., Kim, Y.S., Yuan, J.P., Petralia, R.S., Worley, P.F., and Linden, D.J. (2003). Activation of the TRPC1 cation channel by metabotropic glutamate receptor mGluR1. Nature 426, 285-291.

Kishimoto, Y., Fujimichi, R., Araishi, K., Kawahara, S., Kano, M., Aiba, A., and Kirino, Y. (2002). mGluR1 in cerebellar Purkinje cells is required for normal association of temporally contiguous stimuli in classical conditioning. Eur J Neurosci 16, 2416-2424.

Kitano, J., Nishida, M., Itsukaichi, Y., Minami, I., Ogawa, M., Hirano, T., Mori, Y., and Nakanishi, S. (2003). Direct interaction and functional coupling between metabotropic glutamate receptor subtype 1 and voltage-sensitive Cav2.1 Ca2+ channel. J Biol Chem 278, 25101-25108.

Knopfel, T. (2007). Two new non-competitive mGlu1 receptor antagonists are potent tools to unravel functions of this mGlu receptor subtype. Br J Pharmacol 151, 723-724.

Knopfel, T., and Grandes, P. (2002). Metabotropic glutamate receptors in the cerebellum with a focus on their function in Purkinje cells. Cerebellum 1, 19-26.

Kozlov, A.S., McKenna, F., Lee, J.H., Cribbs, L.L., Perez-Reyes, E., Feltz, A., and Lambert, R.C. (1999). Distinct kinetics of cloned T-type Ca2 + channels lead to differential Ca2 + entry and frequencydependence during mock action potentials. Eur J Neurosci 11, 4149-4158.

Kuo, C.C., Chen, W.Y., and Yang, Y.C. (2004). Block of tetrodotoxin-resistant Na+ channel pore by multivalent cations: gating modification and $\mathrm{Na}+$ flow dependence. J Gen Physiol 124, 27-42.

Lee, J.H., Gomora, J.C., Cribbs, L.L., and Perez-Reyes, E. (1999). Nickel block of three cloned T-type calcium channels: low concentrations selectively block alpha1H. Biophys J 77, 3034-3042.

Loewenstein, Y., Mahon, S., Chadderton, P., Kitamura, K., Sompolinsky, H., Yarom, Y., and Hausser, M. (2005). Bistability of cerebellar Purkinje cells modulated by sensory stimulation. Nat Neurosci 8 , 202-211.

Lopez-Bendito, G., Shigemoto, R., Lujan, R., and Juiz, J.M. (2001). Developmental changes in the localisation of the mGluR 1alpha subtype of metabotropic glutamate receptors in Purkinje cells. Neuroscience 105, 413-429.

Maejima, T., Oka, S., Hashimotodani, Y., Ohno-Shosaku, T., Aiba, A., Wu, D., Waku, K., Sugiura, T., and Kano, M. (2005). Synaptically driven endocannabinoid release requires Ca2+-assisted metabotropic glutamate receptor subtype 1 to phospholipase Cbeta4 signaling cascade in the cerebellum. J Neurosci 25, 6826-6835.

McDonough, S.I., and Bean, B.P. (1998). Mibefradil inhibition of T-type calcium channels in cerebellar purkinje neurons. Mol Pharmacol 54, 1080-1087.

McKay, B.E., McRory, J.E., Molineux, M.L., Hamid, J., Snutch, T.P., Zamponi, G.W., and Turner, R.W. (2006). $\mathrm{Ca}(\mathrm{V}) 3$ T-type calcium channel isoforms differentially distribute to somatic and dendritic compartments in rat central neurons. Eur J Neurosci 24, 2581-2594.

McRory, J.E., Santi, C.M., Hamming, K.S., Mezeyova, J., Sutton, K.G., Baillie, D.L., Stea, A., and Snutch, T.P. (2001). Molecular and functional characterization of a family of rat brain T-type calcium channels. J Biol Chem 276, 3999-4011.

Meacham, C.A., White, L.D., Barone, S., Jr., and Shafer, T.J. (2003). Ontogeny of voltage-sensitive calcium channel alpha( 1 A ) and alpha(1E) subunit expression and synaptic function in rat central nervous system. Brain Res Dev Brain Res 142, 47-65.

Molineux, M.L., McRory, J.E., McKay, B.E., Hamid, J., Mehaffey, W.H., Rehak, R., Snutch, T.P., Zamponi, G.W., and Turner, R.W. (2006). Specific T-type calcium channel isoforms are associated with distinct burst phenotypes in deep cerebellar nuclear neurons. Proc Natl Acad Sci U S A 103, 5555-5560.

Nakamura, T., Barbara, J.G., Nakamura, K., and Ross, W.N. (1999). Synergistic release of Ca2+ from IP3-sensitive stores evoked by synaptic activation of mGluRs paired with backpropagating action potentials. Neuron 24, 727-737.

Pereverzev, A., Mikhna, M., Vajna, R., Gissel, C., Henry, M., Weiergraber, M., Hescheler, J., Smyth, N., and Schneider, T. (2002). Disturbances in glucose-tolerance, insulin-release, and stress-induced hyperglycemia upon disruption of the $\mathrm{Ca}(\mathrm{v}) 2.3$ (alpha 1E) subunit of voltage-gated $\mathrm{Ca}(2+)$ channels. Mol Endocrinol 16, 884-895.

Perez-Reyes, E. (2003). Molecular physiology of low-voltage-activated t-type calcium channels. Physiol Rev 83, 117-161.

Petrenko, A.B., Tsujita, M., Kohno, T., Sakimura, K., and Baba, H. (2007). Mutation of alpha1G T-type calcium channels in mice does not change anesthetic requirements for loss of the righting reflex and minimum alveolar concentration but delays the onset of anesthetic induction. Anesthesiology 106, 11771185.

Pouille, F., Cavelier, P., Desplantez, T., Beekenkamp, H., Craig, P.J., Beattie, R.E., Volsen, S.G., and Bossu, J.L. (2000). Dendro-somatic distribution of calcium-mediated electrogenesis in purkinje cells from rat cerebellar slice cultures. J Physiol 527 Pt 2, 265-282.

Randall, A.D., and Tsien, R.W. (1997). Contrasting biophysical and pharmacological properties of Ttype and R-type calcium channels. Neuropharmacology 36, 879-893.

Roth, A., and Hausser, M. (2001). Compartmental models of rat cerebellar Purkinje cells based on simultaneous somatic and dendritic patch-clamp recordings. J Physiol 535, 445-472.

Sacco, T., and Tempia, F. (2002). A-type potassium currents active at subthreshold potentials in mouse cerebellar Purkinje cells. J Physiol 543, 505-520.

Shigemoto, R., Nakanishi, S., and Mizuno, N. (1992). Distribution of the mRNA for a metabotropic glutamate receptor (mGluR1) in the central nervous system: an in situ hybridization study in adult and developing rat. J Comp Neurol 322, 121-135.

Svoboda, K., Tank, D.W., and Denk, W. (1996). Direct measurement of coupling between dendritic spines and shafts. Science 272, 716-719.

Swensen, A.M., and Bean, B.P. (2003). Ionic mechanisms of burst firing in dissociated Purkinje neurons. J Neurosci 23, 9650-9663.

Tai, C., Kuzmiski, J.B., and MacVicar, B.A. (2006). Muscarinic enhancement of R-type calcium currents in hippocampal CA1 pyramidal neurons. J Neurosci 26, 6249-6258.

Takechi, H., Eilers, J., and Konnerth, A. (1998). A new class of synaptic response involving calcium release in dendritic spines. Nature 396, 757-760.

Talley, E.M., Cribbs, L.L., Lee, J.H., Daud, A., Perez-Reyes, E., and Bayliss, D.A. (1999). Differential distribution of three members of a gene family encoding low voltage-activated (T-type) calcium channels. J Neurosci 19, 1895-1911.

Tanaka, J., Nakagawa, S., Kushiya, E., Yamasaki, M., Fukaya, M., Iwanaga, T., Simon, M.I., Sakimura, K., Kano, M., and Watanabe, M. (2000). Gq protein alpha subunits Galphaq and Galpha11 are localized at postsynaptic extra-junctional membrane of cerebellar Purkinje cells and hippocampal pyramidal cells. Eur J Neurosci 12, 781-792.

Tempia, F., Alojado, M.E., Strata, P., and Knopfel, T. (2001). Characterization of the mGluR(1)mediated electrical and calcium signaling in Purkinje cells of mouse cerebellar slices. J Neurophysiol 86, 1389-1397.

Topolnik, L., Azzi, M., Morin, F., Kougioumoutzakis, A., and Lacaille, J.C. (2006). mGluR1/5 subtypespecific calcium signalling and induction of long-term potentiation in rat hippocampal oriens/alveus interneurones. J Physiol 575, 115-131.

Welsby, P.J., Wang, H., Wolfe, J.T., Colbran, R.J., Johnson, M.L., and Barrett, P.Q. (2003). A mechanism for the direct regulation of T-type calcium channels by Ca2+/calmodulin-dependent kinase II. J Neurosci 23, 10116-10121.

Williams, S.R., Christensen, S.R., Stuart, G.J., and Hausser, M. (2002). Membrane potential bistability is controlled by the hyperpolarization-activated current $\mathrm{I}(\mathrm{H})$ in rat cerebellar Purkinje neurons in vitro. J Physiol 539, 469-483.

Williams, S.R., Toth, T.I., Turner, J.P., Hughes, S.W., and Crunelli, V. (1997). The 'window' component of the low threshold Ca2+ current produces input signal amplification and bistability in cat and rat thalamocortical neurones. J Physiol 505 (Pt 3), 689-705.

Wolfe, J.T., Wang, H., Howard, J., Garrison, J.C., and Barrett, P.Q. (2003). T-type calcium channel regulation by specific G-protein betagamma subunits. Nature 424, 209-213.

Womack, M.D., and Khodakhah, K. (2004). Dendritic control of spontaneous bursting in cerebellar Purkinje cells. J Neurosci 24, 3511-3521.

Zamponi, G.W., Bourinet, E., and Snutch, T.P. (1996). Nickel block of a family of neuronal calcium channels: subtype- and subunit-dependent action at multiple sites. J Membr Biol 151, 77-90.

## 4 DISCUSSION

### 4.1 Overall significance and strengths

### 4.1.1 T-type calcium channel modulation in a heterologous system

The molecular and physiological analysis of T-type $\mathrm{Ca}^{2+}$ channel modulation is a relatively recent aspect of the $\mathrm{Ca}^{2+}$ channel field. A major objective of this thesis was to contribute to this area by systematically investigating the effects of two types of neuronal GPCRs, mAChRs (Chapter 2) and mGluR1 (Chapter 3, Figure 1), concerning their ability to modulate the three main types of recombinant T-type $\mathrm{Ca}^{2+}$ channels ( $\mathrm{Ca}_{v} 3.1, \mathrm{Ca}_{\mathrm{v}} 3.2$ and $\mathrm{Ca}_{\mathrm{v}} 3.3$ ). An initial heterologous approach was chosen in order to eliminate complications associated with the multiple mAChR, mGluR and T-type $\mathrm{Ca}^{2+}$ channel subtypes co-expressed in many native cell types, an issue further complicated by a lack of subtypespecific pharmacological antagonists and possible space-clamping concerns in many native neuronal systems. Most studies examining intracellular modulators of T-type channel activity, such as G $\beta \gamma$ and CAMKII, have not characterized the effects on all three Cav 3 subtypes (Welsby et al., 2003; Wolfe et al., 2003). Utilizing available channel and receptor clones, this is the first study to characterize the effects of GPCR activation on all three major T-type $\mathrm{Ca}^{2+}$ channel isoforms. Our demonstration of the selective inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.3$ currents by $\mathrm{G}_{\mathrm{q} / 11}$-coupled mAChR and mGluR1 receptors provides the first evidence for the specific modulation of a T-type isoform other than $\mathrm{Ca}_{\mathrm{v}} 3$.2. This specific interaction may contribute to $\mathrm{Ca}_{\mathrm{v}} 3.3$ channels playing unique roles in signaling and excitability within the nervous system. The $\mathrm{Ga}_{\mathrm{q}}$-mediated inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.3$ channels is also unique in that it does not involve PLC activity or its downstream signaling elements. Further study of this pathway may shed light on a novel mode of voltage-independent inhibition that could exist for other classes of $\mathrm{Ca}^{2+}$ channels (see section 4.2.2 for further discussion). Although the specific modulator that physically binds to $\mathrm{Ca}_{\mathrm{v}} 3.3$ channels has yet to be elucidated, the regions of the $\mathrm{Ca}_{\mathrm{v}} 3.3$ channel that are involved in this interaction have been conclusively identified. As discussed in Chapter 2, two separate regions of the $\mathrm{Ca}_{\mathrm{v}} 3.3$ are both necessary and sufficient for the inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.3$ by $\mathrm{G}_{\mathrm{q} / 11}$-coupled M 1 receptors. These regions do not contain the domain II-III linker "hotspot" of Ca, 3.2 modulation (Kim et al., 2006; Welsby et al., 2003; Wolfe et al., 2003), indicating that novel channel regions are involved in this T-type inhibition. Finally, the potentiation of $\mathrm{Ca}_{\mathrm{v}} 3.1$ and $\mathrm{Ca}_{\mathrm{v}} 3.2$ channels by $\mathrm{G}_{\mathrm{q} / 11}$-coupled M 1 and mGluR1 receptors in a percentage of HEK cells indicated that a separate modulatory pathway may exist for these T-type isoforms. Further exploration into the mechanism and physiological relevance of this potentiation was examined in cerebellar PCs.

### 4.1.2 T-type calcium channel modulation in cerebellar Purkinje cells

The basic biophysical properties of the T-type $\mathrm{Ca}^{2+}$ channels expressed in cerebellar PCs has been previously determined (Isope and Murphy, 2005), although their molecular composition, physiological roles and potential modulation by GPCRs remained unknown (reviewed in (Cavelier and Bossu, 2003)). The present study is the first to demonstrate that cerebellar PC T-type currents consist of $\mathrm{Ca}_{\mathrm{v}} 3.1$ channels. Furthermore, we show that the physiological stimulation of PF bundles causes the activation of T-type currents at synapses within the distal dendrites of PCs. In the first example of Ttype $\mathrm{Ca}^{2+}$ current modulation within the cerebellum, we found that activation of mGluR1 results in a robust potentiation of T-type currents in both the proximal dendrites and spines of PCs. This augmentation of T-type current involves both an increase in maximal conductance and a hyperpolarizing shift in the voltage-dependence of activation and occurs through a unique signaling pathway characteristic of mGluR1 signaling in PC dendrites (Canepari and Ogden, 2003). The effects of the mGluR1-mediated T-type stimulation on local integration and overall excitability within PCs will be discussed in greater detail in section 4.3.1. Our study on the modulation of PC T-type currents is strengthened by the use of multiple techniques including ultrafast two photon $\mathrm{Ca}^{2+}$ imaging, genetics ( $\mathrm{Ca}_{\mathrm{v}} 3.1$ and $\mathrm{Ca}_{\mathrm{v}} 2.3 \mathrm{KO}$ mice), multiple forms of electrophysiological recordings (voltage clamp, current clamp, and synaptic stimulation), and pharmacological manipulations. The combination of these strategies allowed us to largely overcome technical challenges such as a lack of specific T-type antagonists and potential space clamping issues.

### 4.2 Specific inhibition of $\mathrm{Ca}_{\mathbf{v}} 3.3$ channels by $\mathbf{G} \alpha_{q / 11}$-coupled receptors

### 4.2.1 Working hypothesis

In Chapter 2, we demonstrated for the first time that Ca 3 3.3 T-type channels can be specifically inhibited by $\mathrm{G} \alpha_{q / 11}$-coupled mAChRs when expressed in the heterologous HEK system. The elucidation of a native neuronal system where this modulation might exist is hindered by the lack of identification of $\mathrm{Ca}_{\mathrm{v}} 3.3$ functional expression in the CNS and PNS. Although many neuronal types have been shown to robustly express Cav 3.3 mRNA (Talley et al., 1999) and protein (McKay et al., 2006), the contribution of $\mathrm{Ca}_{\mathrm{v}} 3.3$ channels to functional native T-type currents has not been determined. Unlike the $\mathrm{Ca}_{\mathrm{v}} 3.1$ and $\mathrm{Ca}_{\mathrm{v}} 3.2$ isoforms, KO mice have yet to be generated for the $\mathrm{Ca}_{\mathrm{v}} 3.3$ isoform, and there are no $\mathrm{Ca}_{\mathrm{v}} 3.3-$ specific antagonists that have been identified to date. The development of these tools along with the use of RNAi knock-down approaches are needed to investigate the impacts of $\mathrm{Ca}_{\mathrm{v}} 3.3$-mediated signaling and excitability in the brain (proposals for future research can be found throughout this discussion chapter).

The best candidate for a neuronal population expressing Cav 3.3 -mediated T-type currents is the thalamic nRT neuron. It has been known for some time that T-type currents in nRT neurons have much
slower activation and inactivation kinetics than other "typical" native T-type currents, such as those observed in thalamic TC cells (Huguenard and Prince, 1992). Along with these unique biophysical properties, the T-type currents in acutely dissociated nRT neurons are only partially blocked by $200 \mu \mathrm{M}$ $\mathrm{Cd}^{2+}$ and $100 \mu \mathrm{M} \mathrm{Ni}^{2+}$ (Huguenard and Prince, 1992), suggestive of the presence of $\mathrm{Ca}_{v} 3.3$ channels rather than $\mathrm{Ca}_{\mathrm{v}} 2.3, \mathrm{Ca}_{\mathrm{v}} 3.1$, or $\mathrm{Ca}_{\mathrm{v}} 3.2$ channels (Fox et al., 1987; Lee et al., 1999; Tai et al., 2006; Zamponi et al., 1996). However, Todorovic and colleagues have recently used an elegant combination of whole-cell patch, nucleated patch, and cell-attached recordings on nRT cells within thalamic slices to demonstrate a small somatic T-type current with fast inactivation kinetics and a larger dendritic T-type current that has significantly slower inactivation kinetics (Joksovic et al., 2005). Currents in somatic nRT nucleated patches were 10 -fold smaller in amplitude and over two-fold faster in inactivation kinetics compared to whole-cell currents (Joksovic et al., 2005). The same voltage-dependence of activation between T-type currents in nucleated patch and whole-cell recordings indicated that the slower wholecell currents were not just poorly clamped larger dendritic currents of the same subtype. Furthermore, cell-attached recordings on proximal dendrites and the soma of nRT cells verified the slower inactivation rates of dendritic T-type currents. Pharmacology experiments indicated that the faster somatic currents were over two-fold more sensitive to $\mathrm{Ni}^{2+}$ than the slowly inactivating dendritic T-type currents (Joksovic et al., 2005). This combination of biophysical and pharmacological evidence combined with the detection of $\mathrm{Ca}_{\mathrm{v}} 3.2$ and $\mathrm{Ca}_{\mathrm{v}} 3.3 \mathrm{mRNA}$ in nRT cells (Talley et al., 1999) suggests that $\mathrm{Ca}_{\mathrm{v}} 3.3$ channels primarily underlie dendritic T-type currents and $\mathrm{Ca}_{\mathrm{v}} 3.2$ currents underlie the smaller somatic currents in nRT cells (Joksovic et al., 2005; Lee et al., 1999; McRory et al., 2001). The reduction in Ttype current amplitude in nRT neurons from $\mathrm{Ca}_{\mathrm{v}} 3.2 \mathrm{KO}$ mice verifies the contribution of $\mathrm{Ca}_{\mathrm{v}} 3.2$ channels (Joksovic et al., 2006), but the contribution of $\mathrm{Ca}_{v} 3.3$ channels has yet to be directly demonstrated. However, the resistance to redox potentiation, slower inactivation kinetics, and robust current amplitude of the remaining T-type currents in $\mathrm{Ca}_{\mathrm{v}} 3.2 \mathrm{KO}$ mice (Joksovic et al., 2006) further implicates the functional presence of $\mathrm{Ca}_{\mathrm{v}} 3.3$ channels in these cells.

Ascending cholinergic systems carry sensory information from the PNS to the cortex via relay centers in the thalamus. The molecular mechanism of relaying this information also regulates the sleepwake cycle. When awake, ascending cholinergic systems are activated and cause the resting membrane potential of TC cells to become depolarized. This depolarization is primarily due to the reduction in a relatively linear, non-voltage-dependent potassium leak current (McCormick, 1992). The cholinergicmediated hyperpolarization of nRT neurons also reduces the GABAergic hyperpolarization of TC neurons (reviewed in (Steriade, 2004)). In the depolarized state, TC T-type currents are no longer deinactivated and a switch from rhythmic burst firing to tonic firing occurs, preventing the generation of slow sleep spindle waves (McCormick, 1992). However, the possible direct effect of ascending cholinergic systems on thalamic T-type currents has not been explored. nRT cells have been shown to
express high levels of muscarinic acetylcholine M2 and M3 receptors (Levey et al., 1994; Plummer et al., 1999). Activation of M3 receptors usually results in neuronal depolarization (reviewed in (Jones, 1993)), so their linkage to the hyperpolarizing inhibitory muscarinic response observed in nRT cells is currently unknown (Plummer et al., 1999).

Based upon our observations in the heterologous system, activation of $\mathrm{G} \alpha_{q / 11}$-coupled M3 receptors in nRT dendrites may cause the inhibition of native $\mathrm{Ca}_{\mathrm{v}} 3.3$ currents and a concomitant increase in inactivation kinetics. We also found that M2 receptor activation has no effect on $\mathrm{Ca}_{\mathrm{v}} 3.3$ currents, although the effect of M 2 and M 3 receptor activation on recombinant $\mathrm{Ca}_{\mathrm{v}} 3.2$ currents has not been tested. Further experiments are needed to test the effects of activation of these mAChRs on Cav 3.1 and $\mathrm{Ca}_{\mathrm{v}} 3.2$ currents expressed in HEK cells. If M3 receptor activation has the same effects on $\mathrm{Ca}_{\mathrm{v}} 3.2$ currents as M1 receptor activation, then it could be hypothesized that activation of M3 receptors by ascending cholinergic systems would cause a potentiation of $\mathrm{Ca}_{\mathrm{v}} 3.2$-mediated somatic nRT currents and an inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.3$-mediated dendritic nRT currents. As these modulatory effects could effectively cancel each other out during whole cell recordings, the combined approach of nucleated patch, dendritic cell-attached, and whole-cell recordings needs to be used to test this hypothesis (Joksovic et al., 2005). The slow kinetics of native T-type currents in nRT neurons enables the generation of slow and prolonged bursts that are required for the $\mathrm{GABA}_{\mathrm{B}}$-mediated hyperpolarization of TC neurons during rhythmic rebound burst sleep spindle oscillations (Huguenard and Prince, 1992). An increase in Cav 3.3 T-type current kinetics by the activation of M3 muscarinic receptors could cause nRT T-type currents to become more "Cav 3.1 -like" and potentially shorten burst firing in nRTs , promoting the transition from thalamic rebound burst firing to tonic firing at higher frequencies (Chemin et al., 2002). Thus, in addition to its depolarizing effects, acetylcholine could act physiologically in a manner similar to exogenous succinimide antiepileptics that reduce burst firing and intrathalamic synchronization through the blockade of nRT T-type currents (Huguenard and Prince, 1992; Pellegrini et al., 1989). This would support our overall hypothesis that the specific inhibition of $\mathrm{Ca} \mathrm{v}_{\mathrm{v}} 3.3$-mediated T-type currents by $\mathrm{G} \alpha_{q / 11^{-}}$ coupled receptors alters electrical excitability and firing rhythms within the nervous system.

### 4.2.2 Possible limitations and weaknesses

Besides the current lack of demonstration of the inhibition of native $\mathrm{Ca}_{\mathrm{v}} 3.3$ currents by $\mathrm{Go}_{q / 11-}$ coupled mAChRs, several other limitations exist in our study on the modulation of T-type channels expressed in HEK cells. Further experiments are still needed to identify the specific amino acid residues within the $\mathrm{Ca}_{\mathrm{v}} 3.3$ channel that are involved in the $\mathrm{G}_{\alpha_{q / 11}}$-mediated inhibition. However, before this can be achieved, the intracellular signaling molecule that links $\mathrm{G}_{\mathrm{q} / 11}$ activity to $\mathrm{Ca}_{\mathrm{v}} 3.3$ inhibition needs to be
identified. One possibility is that the $\mathrm{G}_{\mathrm{q} / 11}$ molecule itself directly binds to and inhibits $\mathrm{Ca}_{\mathrm{v}} 3.3$ channels.

A commonly held view concerning $\mathrm{Ca}^{2+}$ channel modulation occuring through $\mathrm{G} \alpha_{q / 11}$-coupled receptor pathways is that depletion of membrane $\mathrm{PIP}_{2}$ through the activation of PLC underlies the voltage-independent inhibition of Ca channels (and other ion channels; reviewed in (Gamper and Shapiro, 2007; Suh and Hille, 2005)). However, several studies have now demonstrated that $\mathrm{G} \alpha_{q / 11}$ binding itself can also directly modulate ion channel activity. Similar to the $\mathrm{G} \alpha_{q / 11}$-mediated inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.3$ channels, the substance P-induced inhibition of Kir3 G-protein coupled inward rectifier $\mathrm{K}^{+}$ channels is rapid, involves $\mathrm{G} \alpha_{q / 11}$-protein signaling, and is independent of membrane $\mathrm{PIP}_{2}$ depletion (Koike-Tani et al., 2005). Interestingly, $\mathrm{G} \mathrm{\alpha}_{\mathrm{q}}$ has been shown to co-immunoprecipitate with Kir3.2 (Koike-Tani et al., 2005) and recent GST pull-down assays have conclusively demonstrated that $\mathrm{G} \alpha_{\mathrm{q}}$ binds directly and specifically to the N-terminus (likely between amino acids $81-90$ ) of Kir3.1, Kir3.2, and Kir3.4 channels (Kawano et al., 2007). The same direct $\mathrm{Ga}_{\mathrm{q}}$-channel binding interaction that is independent of PLC activity has also been shown to underlie the inhibition of two-pore domain $\mathrm{K}^{+}$leak channels (TASK-1 and TASK-2) by $\mathrm{G} \alpha_{q / 11}$-coupled receptors (Chen et al., 2006).

The direct binding of $\mathrm{G} \alpha_{\mathrm{q}}$ is not limited to $\mathrm{K}^{+}$channels; co-immunoprecipitation experiments have revealed a physical interaction between $\mathrm{G}_{\mathrm{q}}$ and the proximal region of the C -terminus of $\mathrm{Ca}_{\mathrm{v}} 2.2$ N-type channels (Simen et al., 2001). I hypothesize that the inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.3$ channels by $\mathrm{G}_{\mathrm{q} / 111^{-}}$ coupled mAChRs is also mediated by the direct binding of activated $\mathrm{G} \alpha_{q / 11}$ protein. If direct $\mathrm{G} \alpha_{q}$ binding mediates a novel mechanism of voltage-independent inhibition, then identification of specific channel regions and sequences involved in this binding would be essential to the further study of this inhibition pathway for all $\mathrm{Ca}^{2+}$ channel classes. Specific residues within the amino terminus of a GPCR kinase, GRK2, have been demonstrated to be essential for direct binding to $\mathrm{Ga}_{\mathrm{q}}$ (Sterne-Marr et al., 2003). These eight identified residues lay within an RGS homology (RH) domain. Alignment of this identified $\mathrm{Ga}_{\mathrm{q}}$ binding region in GRK2 with the Kir3.2 region implicated in $\mathrm{G} \alpha_{q}$ binding (amino acids 81 to 90 ) and regions within the domain I-II linker of $\mathrm{Ca}_{\mathrm{v}} 3.1$ and $\mathrm{Ca}_{\mathrm{v}} 3.3$ channels demonstrates that several of the residues that are essential for the binding of G $\alpha_{q}$ to GRK2 are conserved or homologous in both Kir3.2 and $\mathrm{Ca}_{\mathrm{v}} 3.3$, but not in $\mathrm{Ca}_{\mathrm{v}} 3.1$ channels (Fig. 4.1). Thus, $\mathrm{Ga}_{q / 11}$ may directly bind to a region within the domain I-II linker of $\mathrm{Ca}_{\mathrm{v}} 3.3$ channels to cause channel inhibition. Future experiments that could test this hypothesis are outlined in section 4.4.3. However, until this $\mathrm{Ga}_{\mathrm{q}}$ binding inhibitory mechanism is demonstrated, inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.3$ channels by intracellular signals downstream of PLC activation can not be conclusively ruled out, as positive controls to ensure pharmacological activity were not performed on all kinase/ phosphatase antagonists used in Chapter 2.


Figure 4.1 - Amino acid sequence alignments.
Comparison of an amino acid sequence in the domain I-II linker of $\mathrm{Ca}_{\mathrm{v}} 3.1$ and $\mathrm{Ca}_{\mathrm{v}} 3.3$ channels (identified by a Clustal alignment) with a region in the N-terminus of GRK2 that forms a RH domain and has specific residues directly demonstrated to be involved in binding to $\mathrm{Ga}_{\mathrm{q}}$ (shown in orange) (SterneMarr et al., 2003). Amino acids \# 81-90 from the C-terminus of the Kir3.1 channels were also compared to the GRK2 sequence, as these Kir3.1 amino acids are also implicated in direct binding to $\mathrm{G} \alpha_{\mathrm{q}}$ (Kawano et al., 2007). Amino acids that are conserved when compared to the GRK2 sequence are labeled red,
while those that are homologous to the GRK2 sequence are labeled yellow. The Cav 3.3 domain I-II linker region has much more sequence homology at critical $\mathrm{Ga}_{\mathrm{q}}$ binding residues than the $\mathrm{Ca}_{\mathrm{v}} 3.1$ domain I-II linker region.

### 4.3 Potentiation of $\mathbf{C a}_{\mathbf{v}} 3.1$ currents by mGluR1

### 4.3.1 Working hypotheses

The potentiation of native $\mathrm{Ca}_{\mathrm{v}} 3.1$ T-type currents by mGluR1 activation could have significant effects on electrical and chemical integration within PC dendritic spines, with the potential to alter plasticity at PF-PC synapses. Synaptic activation of $\mathrm{Ca}^{2+}$ channels in these distal dendritic spines has been shown to underlie $\mathrm{Ca}^{2+}$ signals that are restricted to individual spines (Denk et al., 1995). When PF bundles are densely activated, postsynaptic $\mathrm{Ca}^{2+}$ channels can cause a more widespread dendritic depolarization that underlies an mGluR1-independent form of $\mathrm{Ca}^{2+}$ signaling and PF-LTD (Wang et al., 2000). Other than these two pieces of evidence, almost nothing is known about the composition, localization, modulation and physical interactions of $\mathrm{Ca}^{2+}$ channels within PC distal dendritic spines.

Spines are specialized dendritic outgrowths that can compartmentalize both the electrical and biochemical signals that are produced by synaptic inputs (reviewed in (Bloodgood and Sabatini, 2007)). From an electrical perspective, the passive properties of spines include the attenuation of synaptic potentials, depending on the spine neck geometry (narrower neck $=$ higher resistance $=$ higher filtering), as well as the boosting of local EPSPs due to a high spine head input resistance. The combination of these two opposing passive effects has led many to conclude that spines do not play a significant role as electrical compartments. However, neuronal spines can also function as active electrical structures, with voltage-gated channels that can amplify synaptic EPSPs to boost signals and generate local APs, or conversely, voltage-gated channels that can filter EPSPs and reduce excitatory inputs (reviewed in (Tsay
and Yuste, 2004)). The current state of understanding on this function of spines is that "the electrical role of spines remains open and appears to us a rich field - one in which the key experiments have yet to be done" (Tsay and Yuste, 2004). Because of the uncertainty regarding their electrical functions, many researchers have concluded that spines act primarily as biochemical compartments. In support of this notion, the diffusion rates of small substances between spines and their parent dendrites are around 100 times slower than expected for free diffusion of these substances (reviewed in (Nimchinsky et al., 2002)).

Cerebellar PCs are an interesting model neuron for studying spine function, as they contain a much higher density of spines ( 10 spines $/ \mu \mathrm{m}$ ) than most other neurons and these spines have several unique properties. Unlike hippocampal and neocortical pyramidal neurons, PC spines are not lost when afferent connections are removed (reviewed in (Nimchinsky et al., 2002)), and induction of LTD does not cause a change in spine size or number (Sdrulla and Linden, 2007). Thus, changes in synaptic strength in PC dendritic spines likely involve biochemical changes in signaling rather than morphological changes in spiny synapses. The spines on the distal dendrites of PCs each contain a PFPC synapse and have more slender necks than the short and stubby spines of proximal CF-PC synapses. The tight regulation of $\mathrm{Ca}^{2+}$ signaling within these distal dendritic spines through high expression of $\mathrm{Ca}^{2+}$ buffers (calbindin and parvalbumin), channels, and pumps also contributes to their biochemical compartmentalization. PC spines also contain a smooth ER (SER) that continuously extends into the parent dendrite to form a network capable of linking spine biochemical activity to dendrite activity (reviewed in (Nimchinsky et al., 2002)). In addition to the high biochemical compartmentalization within spines of PC distal dendrites, voltage compartmentalization is several-fold greater in PF-PC spines than hippocampal schaffer collateral-CA1 spines (reviewed in (Nimchinsky et al., 2002)).

Recent experiments have unveiled a complex network of interactions between the transmembrane and intracellular peptides that underlie the highly localized mGluR1 signaling within PC distal dendritic spines (Finch and Augustine, 1998; Takechi et al., 1998). Within PF-PC spines, mGluR1 has been shown to be physically linked to TRPC1 and $\mathrm{Ca}_{\mathrm{v}} 2.1$ channels in the plasma membrane (Kim et al., 2003; Kitano et al., 2003; Kulik et al., 2004) as well as PLC $\beta 4$ and $\mathrm{IP}_{3}$ receptors ( $\mathrm{IP}_{3} \mathrm{Rs}$ ) in the intracellular compartment (Nakamura et al., 2004; Sandona et al., 2003; Tu et al., 1998) (Fig. 4.2). Many of these signaling elements are tethered to each other through the binding of Homer cytoskeletal proteins; e.g. - $\mathrm{IP}_{3} \mathrm{R}$ linkage to TRPC1 and mGluR1 (Tu et al., 1998; Yuan et al., 2003). Other peptides directly interact such as the binding between the carboxy terminals of mGluR1 and Cav 2.1 (Kitano et al., 2003). Functional studies showing an activation of $\mathrm{BK} \mathrm{K}^{+}$currents through a combination of P-type $\mathrm{Ca}^{2+}$ channel activity and mGluR1 activity in PC spines indicates that this channel may also be closely associated with mGluR1 at the PF-PC synapse (Canepari and Ogden, 2006; Edgerton and Reinhart, 2003; Womack et al., 2004). However, direct evidence has not linked all of these signaling elements
together within a single PC distal dendritic spine, and the functional consequences of many of these interactions are still unknown.


Figure 4.2 - Proposed intracellular signalling microdomains within Purkinje cell spines.
Signaling peptides that have been shown to physically couple to mGluR1 (many mediated by Homer linkages) within PC dendritic spines are indicated by * (reviewed in (Hartmann and Konnerth, 2005; Knopfel and Grandes, 2002)). Activation of mGluR1 in PC spines can increase local intracellular [ $\mathrm{Ca}^{2+}$ ] through $\mathrm{IP}_{3}$-mediated release from the smooth ER and through the activation of the sEPSC, which is partially mediated by TRPC1. The depolarization-induced activation of $\mathrm{Ca}_{\mathrm{v}} 3.1$ and $\mathrm{Ca}_{\mathrm{v}} 2.1 \mathrm{Ca}^{2+}$ channels is also implicated in elevating intracellular $\left[\mathrm{Ca}^{2+}\right]$. Very little is known about the modulation of $\mathrm{Ca}^{2+}$ channels by mGluR1 within dendritic spines. Our pharmacological experiments have indicated that the signaling elements shown in red are not required for the potentiation of T-type currents (Cav 3.1 ) by mGluR1 activation. For example, blocking phospholipase C (PLC) activity or its downstream signals of protein kinase $\mathrm{C}(\mathrm{PKC})$ or $\mathrm{IP}_{3}$ receptor $\left(\mathrm{IP}_{3} \mathrm{R}\right)$ activation had no significant affect on the mGluR1mediated T-type stimulation. Dashed lines represent putative signaling pathways that have not been completely explored. All elements in green are required for the stimulation of T-type currents via mGluR1 activity, which is attenuated when these signals are blocked with pharmacological antagonists. As shown, tyrosine phosphatases are known to also be required for the mGluR1-mediated activation of the sEPSC and the BK $\mathrm{K}^{+}$current in PCs, but these channels' activity are not required for the T-type effect. The calcium-dependency of the T-type stimulation effect is not due to sEPSC or $\mathrm{IP}_{3} \mathrm{R}$ activity alone, but could be due to calcium influx through a combination of these channels acting within the putative signaling microdomain. Many other intracellular scaffolding proteins (such as Homer), signaling proteins (such as arachidonic acid), and ion channels (such as AMPA receptors) that are present in PC distal dendritic spines are omitted for simplicity.

I hypothesize that $\mathrm{Ca}_{\mathrm{v}} 3.1$ is colocalized with mGluR1 and other signaling proteins at PF-PC spines to form signaling microdomains (Fig. 4.2). Several lines of evidence support an interaction between mGluR1 and $\mathrm{Ca}_{\mathrm{v}} 3.1$ in PC distal spiny microdomains (Chapter 3). Firstly, the mGluR1mediated augmentation of T-type $\mathrm{Ca}^{2+}$ transients was observed in PC spines, but not their parent dendrites, indicating that the modulatory interaction was localized to spines. When blockade of $\mathrm{IP}_{3} \mathrm{Rs}$ was removed, the T-type potentiation spread to the parent dendrites. This is consistent with a mechanism whereby robust mGluR1 activation (bath application of DHPG) can cause $\mathrm{Ca}^{2+}$ release from the continuous sER network that propagates the $\mathrm{Ca}^{2+}$-dependent T-type potentiation into adjacent dendrites and spines. Another key piece of evidence that connects $\mathrm{Ca}_{\mathrm{v}} 3.1$ activity to putative mGluR1 signaling microdomains is the modulation pathway that was identified. Both sEPSC and Cav 3.1 channels are activated/ potentiated by an mGluR1 signaling pathway that is dependent on G-protein and tyrosine phosphatase activity but is independent of the activation of PLC or its downstream effectors (Fig. 4.2). Thus, I propose that activation of this unique mGluR1-mediated pathway can increase intracellular $\mathrm{Ca}^{2+}$ levels within PF-PC spiny microdomains through the activation of both voltage-dependent (Cav 3.1 ) and independent (sEPSC) membrane $\mathrm{Ca}^{2+}$ channels. Consistent with this notion, repetitive activation of PF bundles caused an mGluR1-mediated increase in $\mathrm{Ca}^{2+}$ transients that was localized to specific distal dendrites (Chapter 3). These $\mathrm{Ca}^{2+}$ transients were severely reduced or abolished in $\mathrm{Ca}_{\mathrm{v}} 3.1 \mathrm{KO}$ mice, indicating that $\mathrm{Ca}_{\mathrm{v}} 3.1$ channels underlie the primary mGluR1-mediated $\mathrm{Ca}^{2+}$ signal in PF-PC spines under our experimental conditions. The fact that the above experiments were performed at a near physiological temperature $\left(32^{\circ} \mathrm{C}\right)$ on older rats ( P 16 to P 30 ) also demonstrates that the mGluR1mediation potentiation of $\mathrm{Ca}_{\mathrm{v}} 3.1$ currents occurs during physiological synaptic integration within mature PCs.

If the synaptic activation of mGluR1 in PF-PC synapses causes the potentiation of postsynaptic T-type channels, it is also possible that the $\mathrm{Ca}^{2+}$ currents flowing through these channels partially mediate the supralinear $\mathrm{Ca}^{2+}$ signals that underlie PF-LTD. A role for $\mathrm{Ca}^{2+}$ channels in single spine plasticity has been demonstrated in hippocampal CA1 neurons, where the activity of R -type $\mathrm{Ca}^{2+}$ channels is modulated by postsynaptic depolarizations and impacts EPSP amplitude for tens of minutes (Yasuda et al., 2003). In our experiments it is interesting to note that the bath application of exogenous DHPG that potentiated T-type currents has recently been shown by Sdrulla and Linden to induce a robust chemically-evoked form of LTD that occurs on a similar timescale as seen for our T-type modulation (Sdrulla and Linden, 2007). Thus, T-type currents are potentiated under experimental conditions that elicit PF-LTD. The question that remains is whether this mGluR1-mediated potentiation of T-type currents is necessary to induce any forms of physiological PF-LTD. A study of PF-LTD induced by the concurrent activation of both PF and CF inputs demonstrated that LTD induced by sparse activation of PFs is restricted to single spines, dependent upon mGluR1 signaling, and greatest when PF activation
precedes CF activation (Wang et al., 2000). These observations are consistent with a (simplified) hypothetical mechanism of LTD whereby PF inputs activate mGluR1 to potentiate T-type currents and slowly depolarize the spine through the sEPSC. This would be followed by further CF-mediated depolarization which allows the membrane potential in the spines to reach threshold for activation of the now-potentiated T-type currents, providing the necessary $\mathrm{Ca}^{2+}$ influx to induce AMPAR internalization.

In section 4.4.3, I outline future experiments that could elucidate the contribution of $\mathrm{Ca}_{\mathrm{v}} 3.1$ in integration and plasticity at dendritic spine microdomains of the PF-PC synapse. Besides these experiments, future experiments using local PF synaptic activation, high resolution two-photon imaging, specific channel/receptor antagonists, conditional KO mice and voltage-sensitive indicators will be required to determine the individual roles that the other signaling elements (e.g. - sEPSC, Cav 2.1 , PLC $\beta 4, \mathrm{IP}_{3} \mathrm{R}$, etc...) perform within these spines. Although I hypothesize that all of these elements are co-localized within spine compartments, it is likely that only a subset of these components will be activated for a given physiological response. Alternatively, it is also possible that mGluR1 is specifically coupled to different effectors and signaling elements in different regions of the PC distal dendritic tree.

Besides acting as a putative coincidence detector in PF-LTD, the activation and potentiation of T-type $\mathrm{Ca}^{2+}$ channels in PC dendritic spines could also serve to boost or filter synaptic inputs. As we showed in Chapter 3, a temporal summation of high frequency EPSPs could result in the opening of $\mathrm{Ca}_{\mathrm{v}} 3.1$ channels in dendritic spines. This T-type channel opening could cause further depolarization that opens nearby $\mathrm{Ca}_{\mathrm{v}} 2.1$ channels to further boost the depolarizing signal. Alternatively, the opening of Ttype channels could provide the $\mathrm{Ca}^{2+}$ signal necessary to activate nearby $\mathrm{BK} \mathrm{K}^{+}$channels and induce a hyperpolarization that filters the EPSP. As PCs are spontaneously active, with periods of tonic and burst firing separated by quiescent periods, the potentiation of T-type currents by mGluR1 could be crucial in glutamate-mediated alterations of PC excitability states as well as the timing of firing (Williams et al., 2002; Womack and Khodakhah, 2002; Womack and Khodakhah, 2004).

A novel potential role for the stimulation of T-type currents in dendritic spines is the dendritic release of glutamate for both retrograde and autocrine signaling. T-type currents have been shown to underlie spontaneous synaptic $\mathrm{Ca}^{2+}$ transients that are either localized to individual spines or initiate a more widespread low threshold $\mathrm{Ca}^{2+}$ spike at the large dendrodendritic spiny synapses of olfactory bulb granule cells (Egger et al., 2003, 2005). Preliminary experiments indicate that these T-type currents underlie a "presynaptic" dendritic $\mathrm{Ca}^{2+}$ signal that results in the AP-independent release of GABA to retrogradely inhibit the adjacent mitral and tufted output cells of the dendrodendritic synapses (Egger et al., 2003). Exciting new studies indicate that a similar mechanism of dendritic neurotransmitter release may exist in cerebellar PCs. In PF-PC synapses of nearly mature (P18 to P22) rats, glutamate has been shown to be released from PC spines through an mGluR1-mediated mechanism. The released glutamate
binds to kainite receptors on the PF terminals and induces a short-term suppression of excitation that is followed by a potentiation phase (Crepel and Daniel, 2007). Unlike the suppression of excitation, the potentiation is thought to be mediated by a postsynaptic PC mechanism (Crepel and Daniel, 2007). Another very recent study has shown that low frequency CF stimulation or direct somatic depolarization of PCs causes dendritic glutamate release through a classical SNARE-dependent vesicular release mechanism that is usually observed for neurotransmitter release at presynaptic axons terminals (Duguid et al., 2007). This dendritic glutamate release from PCs has subsequently been shown to be dependent on $\mathrm{Ca}^{2+}$ channel activity and results in the autocrine activation of mGluR1 receptors, leading to the release of inhibitory endocannabinoids as well as the activation of the mGluR1-mediated sEPSC (Duguid et al., 2007; Shin et al., 2008). The involvement of mGluR1 in both the release and autocrine action of glutamate combined with the requirement of $\mathrm{Ca}^{2+}$ channel-mediated $\mathrm{Ca}^{2+}$ influx for dendritic glutamate release indicates that the potentiation of T-type $\mathrm{Ca}^{2+}$ channels by mGluR1 could be a critical component for both of these mechanisms. In fact, the potentiation of T-type currents by autocrine mGluR1 activation might explain the delayed postsynaptic PC potentiation observed after glutamate release (Crepel and Daniel, 2007). Along these lines, I often observed a run-up in T-type current amplitude during successive test depolarizations under the control conditions of voltage-clamp experiments that may be due to this putative autocrine mGluR1 signaling activity (unpublished observations).

The potentiation of T-type currents by mGluR1 is also observed in PC proximal dendrites, suggesting that this modulation could be relevant for CF-PC synaptic functions. Stimulation of CF inputs can cause the activation of mGluR1 and its associated sEPSC (Dzubay and Otis, 2002). Furthermore, evidence is emerging that indicates that T-type currents underlie a low-threshold spike in the proximal dendrites that can propagate to the soma and may contribute to the complex spike (Cavelier et al., 2008) (reviewed in (Cavelier and Bossu, 2003)). Isope and colleagues are currently combining experiments involving $\mathrm{Ca}^{2+}$ imaging, current clamp recordings, and CF stimulation to reveal that the CFmediated $\mathrm{Ca}^{2+}$ signaling in proximal dendritic spines is mainly mediated by $\mathrm{Ca}_{\mathrm{v}} 3.1$ T-type currents in the basal state, while the blockade of a fraction of the $\mathrm{I}_{\mathrm{A}} \mathrm{K}^{+}$currents induces a $\mathrm{CF}-$ mediated $\mathrm{Ca}^{2+}$ spike that is dependent on P-type channel activity and can propagate throughout the dendritic tree (Philippe Isope, personal communication). Modulation of T-type currents and other ionic currents by mGluR1 could participate in this "unlocking" of dendritic $\mathrm{Ca}^{2+}$ spikes, possibly through the generation of low-threshold spikes that overcome the inhibitory $\mathrm{K}^{+}$currents. As mGluR1 activity is strongly implicated in CF pruning in the proximal dendrites (reviewed in (Hashimoto and Kano, 2005)), the potentiation of T-type currents by mGluR1 could also have a functional role in this process.

### 4.3.2 Possible limitations and weaknesses

Because of well-known space clamp concerns, a majority of our experiments were performed on PCs from acute brain slices of young (P8-12) rats and mice. The dendritic arbourization is limited at this developmental age, allowing us to voltage clamp the PCs and accurately measure the biophysical properties of T-type currents before and after activation of mGluR1 (Chapter 3). Climbing fiber and PF synapses are still being formed and eliminated during this developmental window, and so the potentiation of T-type currents by mGluR1 was observed during a time of CF pruning and could have a direct physiological role in this process. Is the observed modulation relevant at mature synapses in fully developed PCs? We performed a final set of experiments on PCs from older rodents (P16 to P30) and found that the $\mathrm{Ca}_{v} 3.1$-mediated T-type $\mathrm{Ca}^{2+}$ transients are potentiated by physiological activation of mGluR1 at mature PF-PC spiny synapses (Figure 3.7). However, indirect contributions of voltage-gated $\mathrm{K}^{+}$or HVA $\mathrm{Ca}^{2+}$ channels towards the mGluR1-mediated T-type potentiation has not been ruled out in these experiments. Similar imaging and synaptic activation experiments need to be performed on adult rodents to verify both the functional expression of T-type currents as well as their potentiation by mGluR1. Cell-attached dendritic recordings with and without DHPG in the pipette could also be used to directly test for the modulation of T-type currents by mGluR1 in adult PCs and to characterize this putative T-type potentiation at the single channel level. These future experiments are especially important since one study demonstrated a lack of T-type currents in both the soma and dendrites of adult guinea pig PCs (Usowicz et al., 1992).

Another limitation of our experiments is that we have not directly demonstrated a specific physiological process involving the mGluR1-induced potentiation of T-type channels. In order to explore the role of this T-type potentiation in specific physiological processes (discussed in section 4.3.1), a more integrative approach that includes the interactions of mGluR1 with various other effectors and ion channels needs to be utilized. Most of our experiments characterized the modulation of T-type currents by mGluR1 in isolation so that the results could be most easily interpreted. Within a physiological context, activation of mGluR1 could involve the simultaneous modulation of various ion channels, including TRPC1 (Kim et al., 2003), BK (Canepari and Ogden, 2006), and P-type channels (Kitano et al., 2003) as well as other modulated channels that remain to be identified (as was the case for T-type channels before our study). To illustrate the difficulties that arise from this complexity, when all ionic currents were left unblocked for our excitability experiments in Figure 3.4, the role of T-type potentiation in the DHPG-induced changes in firing frequency could not be easily analyzed because multiple ion channels that could be modulated by mGluR1 are involved in this interspike interval parameter (Swensen and Bean, 2003). In fact, the effect of DHPG on the firing frequency appeared to be highly variable (unpublished observation), which could be due to the antagonistic modulation of various ion channels by mGluR1. Interpretation of these experiments are also limited by antagonist specificity,
as both mibefradil and $\mathrm{Ni}^{2+}$ can potentially block R-type currents and other HVA currents at the concentrations used (Lee et al., 1999; Randall and Tsien, 1997; Zamponi et al., 1996). Thus, even the stated role of mGluR 1-mediated potentiation of T-type currents in lowering the AP threshold needs to be further confirmed in $\mathrm{Ca}_{\mathrm{v}} 3.1 \mathrm{KO}$ mice.

The first steps toward understanding these multi-variable processes involve thoroughly characterizing the interactions between mGluR1 and all channels known to be involved in PC physiology. Although our study is a good step forward in this task, the work is far from complete. For example, although P-type channels are the predominant $\mathrm{HVA} \mathrm{Ca}^{2+}$ channels expressed in PC dendrites and soma (Mintz et al., 1992; Usowicz et al., 1992) and are implicated in the firing of the complex spike (Watanabe et al., 1998), only incomplete biochemical and imaging experiments have tested for a modulatory interaction with mGluR1 (Kitano et al., 2003). These experiments conclusively showed that mGluR1a physically couples to P-type channels and is co-localized in PC dendrites, making a modulatory interaction both likely and potentially highly relevant (Kitano et al., 2003). Future voltage clamp experiments are needed to directly characterize the effects of mGluR1 activation on P-type currents in PCs. To overcome the obvious space clamp issues that arise when dealing with these large dendritic P-type currents, a first step could involve investigating the interaction in acutely dissociated juvenile PCs, where both P-type currents and mGluR1 are expressed (Lopez-Bendito et al., 2001; Mintz et al., 1992). This could be followed by the more demanding single channel recordings of P-type current modulation in adult PC dendrites. Once all of these modulatory interactions have been characterized, then KO mice and specific pharmacological antagonists can be used more effectively in combination with $\mathrm{Ca}^{2+}$ imaging and electrophysiological recordings to elucidate the roles of mGluR1-ion channel interactions in physiological processes such as local integration, plasticity, excitability, and synaptic pruning.

The intracellular signaling mechanism that directly links mGluR1 activity to the alteration of Ttype biophysical properties is also currently unknown. Our results have shown that the potentiation pathway involves G-protein activation, intracellular $\mathrm{Ca}^{2+}$ signals, and tyrosine phosphatase activity and is independent of the activation of PLC and its downstream signaling pathways (Fig. 4.2). The first step to further explore this signaling pathway would be to determine whether $G \alpha_{q / 11}$ or $G \beta \gamma$ is involved in the potentiation. To test for a role of $\mathrm{G} \alpha_{q / 11}$, a membrane permeable, N -terminal palmitoylated decapeptide (palpeptide) that has been shown to specifically disrupt $\mathrm{G} \alpha_{q / 11}$ function and subsequent modulation of ion channels (Robbins et al., 2006) could be puffed onto the PC before testing for the modulation of T-type currents by DHPG application. To test for a role of G $\beta \gamma$ binding in the modulation, either purified $G \beta \gamma$ or G $\beta \gamma$-specific antibodies could be included within the internal pipette to disrupt normal $\mathrm{G} \beta \gamma$-mediated signaling (Zamponi et al., 1997). However, sufficient time for dialysis of the purified proteins/ antibodies into the distal dendrites would be required before the modulation of DHPG on T-type currents
could be tested. These experiments could be followed by co-immunoprecipitation studies to test whether the implicated G-protein subunit is directly involved in a physical interaction with the modulated $\mathrm{Ca}_{\mathrm{v}} 3.1$ channels.

### 4.4 Conclusions

### 4.4.1 General conclusions

Overall, we have demonstrated that specific T-type $\mathrm{Ca}^{2+}$ channel isoforms are differentially modulated by certain GPCR pathways. Together, this subtype-specific T-type channel-GPCR coupling likely contributes to the unique cellular functions of each of the various T-type calcium channel subtypes. We found that the $\mathrm{Ca}_{v} 3.3$ isoform is specifically inhibited by $\mathrm{G} \alpha_{q / 11}$-coupled receptors with a concomitant robust increase in channel inactivation kinetics. This $G \alpha_{q / 11}$-mediated inhibition involves intracellular interactions with two distinct channel regions that are independent of cytoplasmic signals downstream of PLC activation. I hypothesize that this inhibition of Ca 3.3 channels by $\mathrm{G}_{\mathrm{q} / 11}$-coupled mAChRs could be involved in the cholinergic regulation of thalamic nRT firing patterns during sleepwake transitions. The $\mathrm{Ca}_{\mathrm{v}} 3.1$ isoform is potentiated by mGluR1 activation within both the heterologous HEK system and cerebellar PCs. The mGluR1-mediated T-type potentiation within PCs involved both an increase in maximal currents as well as a hyperpolarizing shift in the voltage-dependence of activation. These effects lead to a decreased AP threshold and resultant increased excitability within PCs. The potentiation of $\mathrm{Ca}_{\mathrm{v}} 3.1$ by mGluR1 was localized to synaptic locations within PC dendrites, and physiological activation of mGluR1 through a burst of PF stimulation induced a robust augmentation of T-type transients. I hypothesize that the potentiation of $\mathrm{Ca}_{\mathrm{v}} 3.1$ currents by mGluR1 could contribute to the coincidence detector and supralinear $\mathrm{Ca}^{2+}$ signals required for cerebellar PF-LTD and motor learning. Finally, through our collaboration with Soong and coworkers we have found that $\mathrm{Ca}_{\mathrm{v}} 3.2$ channels are selectively inhibited by corticotropin-releasing factor receptors through a G $\beta \gamma$-mediated signaling pathway (Appendix 2) that is independent of G $\alpha$ signaling pathways. This inhibitory interaction could be a critical component relevant to both cardiac and neuronal physiology.

### 4.4.2 Possible relevance to human disease

Several human movement disorders, such as episodic ataxia type-2 and spinocerebellar ataxia type-6, are caused by point mutations in the $\mathrm{Ca}_{\mathrm{v}} 2.1$ channel that disrupt normal P/Q-type channel function in the cerebellum (Spacey et al., 2004) (Appendix 3) (reviewed in (Adams and Snutch, 2007)). Similar to that for $\mathrm{P} / \mathrm{Q}$-type $\mathrm{Ca}^{2+}$ channels, we have now demonstrated that $\mathrm{Ca}_{\mathrm{v}} 3.1 \mathrm{~T}$-type channels are functionally expressed in PC dendrites and may be involved in similar processes, such as complex spike firing. From our data I hypothesize that T-type channel dysfunction within PCs could also be involved in
the pathophysiology of movement disorders such as ataxia. In an animal model of a severe movement disorder, leaner mice have a spontaneous mutation in the P/Q-type channel gene which results in ataxia and dyskinesia. Whole-cell and single-channel recordings have demonstrated that the leaner mutation results in a $\sim 60 \%$ reduction in P-type currents within mature cerebellar PCs by reducing the channel open probability (Dove et al., 1998). A recent expression study using a combination of laser-capture microdissections, quantitative RT-PCR and in situ hybridization has demonstrated that $\mathrm{Ca}_{\mathrm{v}} 3.1$ expression is significantly increased in mature PCs of leaner mice (Nahm et al., 2005). In contrast to these results, no change in T-type current is observed in PCs from ataxic tottering mice that have a less severe P-type mutation and phenotype (Erickson et al., 2007). However, experiments in this second study were done on dissociated PCs that lack the dendritic trees where T-type channels are predominantly expressed and cells were taken from mice at a developmental stage (P6 to P15) that is much earlier than when the motor dysfunction symptoms appear (three to four weeks postnatally) (Erickson et al., 2007). The correlation between increased $\mathrm{Ca}_{\mathrm{v}} 3.1$ expression in PCs and ataxia and dyskinesia indicates that aberrant potentiation of T-type currents by mGluR1 could also potentially lead to movement disorder phenotypes. It would be of interest to examine the level of augmentation of Cav 3.1 T-type currents by mGluR1 in leaner mice to determine whether excessive T-type modulation also contributes to the uncoordinated phenotype in this animal model.

The results of recent studies point to the tantalizing possibility that the potentiation of $\mathrm{Ca}_{\mathrm{v}} 3.1$ channels by mGluR1 is also involved in the physiology and pathophysiology of a brain region that is involved in both sleep-wake gating and the genesis of epileptic seizures: the thalamus. The activation of mGluR1 by cortical inputs is thought to be involved in generating the intrinsic TC oscillations that are critical in slow wave ( $<1 \mathrm{~Hz}$ ) sleep rhythms (Hughes et al., 2002). The underlying mechanism of this slow rhythm induction has been shown to involve a reduction in outward linear leak currents by mGluR1 activation. This modulation results in an overlap between the current-voltage relationships of the outward leak currents and inward T-type window currents, creating membrane bistability and the resultant UP and DOWN states of firing that underlie rhythmic oscillations (Hughes et al., 2002; Williams et al., 1997) (reviewed in (Crunelli et al., 2006)). The activation of depolarizing Ih currents during the hyperpolarized DOWN states is what functionally links the two stable resting membrane potential states together to form a continuous slow oscillation (Hughes et al., 2002; Williams et al., 1997). As Cavv 3.1 channels form the dominant functional T-type currents in these cells (Kim et al., 2001) and are co-localized with mGluR1 (reviewed in (Alexander and Godwin, 2006)), their potentiation by mGluR1 could lead to an increase in T-type window conductance that directly contributes to these state transitions and network oscillations. Indeed, one preliminary study indicates that an mGluR1 signaling pathway within TCs regulates T-type channel activity to control the mode of firing (Cheong et al., 2007). Future voltage-clamp experiments should directly test the effects of activating mGluR1 receptors on the
biophysical properties of T-type channels within TCs. These experiments could demonstrate a broader impact of $\mathrm{Ca}_{\mathrm{v}} 3.1$ modulation by mGluR1 in the CNS, especially as "T-type $\mathrm{Ca}^{2+}$ channels, therefore, constitute the single most crucial voltage-dependent conductance that permeates all activities of thalamic neurons during NREM sleep" (Crunelli et al., 2006).

In addition to an induction of sleep rhythms, the potentiation of $\mathrm{Ca}_{\mathrm{v}} 3.1$ currents by mGluR1 within TCs might also be linked to epileptic SWDs under pathological conditions. T-type $\mathrm{Ca}^{2+}$ channels have a well-documented role in epileptic absence seizures and their underlying mechanism of thalamocortical 3-4 Hz oscillations (SWDs) (reviewed in (Khosravani and Zamponi, 2006)). The "cortical focus" theory of absence epilepsy proposes that cortical hyperexcitability initiates these SWDs through the induction of synchronized firing within the thalamus via their glutamatergic connections onto TCs (and nRTs). This initiation appears to involve mGluR1 receptors, as application of mGluR1 antagonists effectively blocked SWD activity in the "lethargic" mouse model of absence epilepsy (reviewed in (Alexander and Godwin, 2006)). Thus, excessive activation of mGluR1 receptors in TCs by hyperexcited cortical efferents could induce thalamic SWD activity through the potentiation of T-type currents. As we have shown that $\mathrm{Ca}_{\mathrm{v}} 3.2$ channels can also be potentiated by mGluR1 activity, this potentiation mechanism could directly increase rhythmic burst firing within both TCs and nRTs. Therefore, future experiments should also test the effects of mGluR1 activation on the biophysical properties of T-type currents within nRTs. The implication of T-type channel activity and potentiation in movement disorders (Nahm et al., 2005), sleep disorders (Anderson et al., 2005), and epileptic activity (Khosravani and Zamponi, 2006) indicates that specific T-type antagonists have the potential to form novel classes of therapeutic compounds to treat these pathological conditions.

### 4.4.3 Future directions

Throughout this discussion I have outlined several experiments and avenues for future research in the field of T-type modulation. Here, I will discuss more detailed future experiments on two independent aspects of T-type modulation: 1) the interactions between $\mathrm{G} \alpha_{q / 11}$-mediated intracellular signals and $\mathrm{Ca}_{\mathrm{v}} 3.3$ channels involved in the GPCR inhibitory effect, and 2) the putative physical coupling between mGluR1, $\mathrm{Ca}_{\mathrm{v}} 3.1$, and other ion channels in PC dendritic microdomains and the potential consequences of these interactions.

Thus far, we have been unable to directly identify the intracellular signal that links $\mathrm{G} \alpha_{q / 11}$ activity to $\mathrm{Ca}_{\mathrm{v}} 3.3$ channel inhibition. I hypothesize that $\mathrm{G} \alpha_{q / 11}$ itself may directly bind to and inhibit these $\mathrm{Ca}^{2+}$ channels and propose testing this notion using a constitutively active $\mathrm{G} \alpha_{q}$ construct that has mutations in two key residues to eliminate coupling to PLC (Chen et al., 2006). Transfection of this PLC activation-deficient $\mathrm{G}_{\mathrm{q}}$ construct into stable $\mathrm{Ca}_{\mathrm{v}} 3.3$-expressing HEK cells is hypothesized to cause
the same decrease in current amplitude and increase in inactivation kinetics as observed in Figure 3.4 if PLC and its downstream signaling pathways (including PIP $_{2}$ depletion) are not required for inhibition. A positive result in this experiment would confirm the results from our pharmacological experiments (Figs. 3.3 and 3.5). A direct inhibitory interaction involving $\mathrm{G} \alpha_{\mathrm{q}}$ could then also be tested by applying purified active $\mathrm{G} \alpha_{q}$-GTP (and inactive $\mathrm{G} \alpha_{q}$-GDP as a negative control) to inside-out macropatches of HEK cells overexpressing $\mathrm{Ca}_{\mathrm{v}} 3.3$. If direct application of active $\mathrm{Ga}_{\mathrm{q}}$ causes $\mathrm{Ca}_{\mathrm{v}} 3.3$ inhibition in these macropatches then single channel recordings on smaller patches could also be used to characterize what T-type single channel properties are altered in this inhibitory interaction. Co-immunoprecipitation experiments using specific antibodies on lysates from HEK cells transfected with both constitutively active $\mathrm{G} \alpha_{q}$ proteins and $\mathrm{Ca}_{\mathrm{v}} 3.3$ channels could demonstrate whether or not a direct physical interaction exists between these two proteins. If successful, GST pull-down assays between specific $\mathrm{Ca}_{\mathrm{v}} 3.3$ regions and $\mathrm{G} \alpha_{q}$ could verify this result and narrow putative interacting regions (Chen et al., 2006; Wolfe et al., 2003). Combining these pull-down assays with electrophysiological recordings on further $\mathrm{Ca}_{\mathrm{v}} 3.1-\mathrm{Ca}_{\mathrm{v}} 3.3$ chimeric channels and then $\mathrm{Ca}_{\mathrm{v}} 3.3$ channels with specific point mutations could demonstrate the channel regions and residues involved in the $\mathrm{Ga}_{\mathrm{q}}$-mediated inhibition. An initial candidate region to be tested would be the domain I-II linker region shown in Figure 4.1. All of these above experiments would help to understand the molecular underpinnings of a putatively novel form of voltage-independent inhibition of $\mathrm{Ca}^{2+}$ channels.

Although mGluR1 activation has been shown to modulate Cav 3.1 T-type currents within PCs, the co-localization between these two proteins in PC dendrites has yet to be directly demonstrated. Combining light and electron microscopy with the staining of cerebellar slices using Cave 3.1 and mGluR1 antibodies could determine the ultrastructural localization of $\mathrm{Ca}_{\mathrm{v}} 3.1$ channels in PC dendrites and spines as well as their potential co-localization with mGluR1 receptors in the perisynaptic regions of PF-PC and CF-PC synapses. Subsequent co-immunoprecipitation experiments with these mGluR1 and Cavv 3.1 antibodies could be used to examine whether the two proteins are physically linked to each other. The mGluR1 receptor is known to be physically coupled to other ion channel effectors, such as TRPC1, $\mathrm{Ca}_{\mathrm{v}} 2.1$ channels, and $\mathrm{IP}_{3} \mathrm{Rs}$, and is proposed here to form signaling microdomains with these proteins in PC dendritic spines (Fig. 4.2). If mGluR1 is shown to bind to $\mathrm{Ca}_{\mathrm{v}} 3.1$ channels, the potential physical interactions between $\mathrm{Ca}_{v} 3.1$ channels and other $\mathrm{Ca}^{2+}$ signaling effectors like TRPC1 or $\mathrm{Ca}_{\mathrm{v}} 2.1$ could also be tested using further co-immunoprecipitation experiments. The relevance of the mGluR1-mediated potentiation of $\mathrm{Ca}_{\mathrm{v}} 3.1$ currents in synaptic plasticity within these spiny microdomains also remains to be tested. Concurrent activation of PF and CF inputs while recording EPSC amplitude could be used to induce and measure PF-LTD. If Cav 3.1 currents are involved in this depression of EPSC amplitude, then puffing on a T-type antagonist (such as $\mathrm{Ni}^{2+}$ ) or a specific $\mathrm{Ca}_{\mathrm{v}} 3.1$ T-type antagonist (when one is finally developed) over the PC dendrites should abolish the initiation and/or maintenance of this PF-LTD.

Alternatively, PF-LTD could be measured and compared between $\mathrm{Ca}_{\mathrm{v}} 3.1 \mathrm{KO}$ and wt mice. This genetic model would also enable the determination of whether $\mathrm{Ca}_{\mathrm{v}} 3.1$ channel activity is required for cerebellar motor learning activities, such as the vestibulo-ocular reflex.

### 4.5 References

Adams, P.J., and Snutch, T.P. (2007). Calcium Channelopathies: voltage-gated calcium channels. In Calcium Signalling and Disease - Molecular Pathology of Calcium, E. Carafoli, and M. Brini, eds. (New York: Springer), pp. 215-251.

Alexander, G.M., and Godwin, D.W. (2006). Metabotropic glutamate receptors as a strategic target for the treatment of epilepsy. Epilepsy Res 71, 1-22.

Anderson, M.P., Mochizuki, T., Xie, J., Fischler, W., Manger, J.P., Talley, E.M., Scammell, T.E., and Tonegawa, S. (2005). Thalamic Cav3.1 T-type Ca2+ channel plays a crucial role in stabilizing sleep. Proc Natl Acad Sci U S A 102, 1743-1748.

Bloodgood, B.L., and Sabatini, B.L. (2007). Ca(2+) signaling in dendritic spines. Curr Opin Neurobiol 17, 345-351.

Canepari, M., and Ogden, D. (2003). Evidence for protein tyrosine phosphatase, tyrosine kinase, and Gprotein regulation of the parallel fiber metabotropic slow EPSC of rat cerebellar Purkinje neurons. J Neurosci 23, 4066-4071.

Canepari, M., and Ogden, D. (2006). Kinetic, pharmacological and activity-dependent separation of two $\mathrm{Ca} 2+$ signalling pathways mediated by type 1 metabotropic glutamate receptors in rat Purkinje neurones. J Physiol 573, 65-82.

Cavelier, P., and Bossu, J.L. (2003). Dendritic low-threshold Ca2+ channels in rat cerebellar Purkinje cells: possible physiological implications. Cerebellum 2, 196-205.

Cavelier, P., Lohof, A.M., Lonchamp, E., Beekenkamp, H., Mariani, J., and Bossu, J.L. (2008). Participation of low-threshold Ca2+ spike in the Purkinje cells complex spike. Neuroreport 19, 299-303.

Chemin, J., Monteil, A., Perez-Reyes, E., Bourinet, E., Nargeot, J., and Lory, P. (2002). Specific contribution of human T-type calcium channel isotypes (alpha(1G), alpha(1H) and alpha(1I)) to neuronal excitability. J Physiol 540, 3-14.

Chen, X., Talley, E.M., Patel, N., Gomis, A., McIntire, W.E., Dong, B., Viana, F., Garrison, J.C., and Bayliss, D.A. (2006). Inhibition of a background potassium channel by Gq protein alpha-subunits. Proc Natl Acad Sci U S A 103, 3422-3427.

Cheong, E., Lee, S., Choi, B.J., Sun, M., Lee, C.J., and Shin, H.S. (2007). Concomitant control of T-type and L-type $\mathrm{Ca} 2+$ channels in thalamocortical neurons by the mGluR1-PLC $\beta 4$ cascade sets the thalamic sensory gating. In Society for Neuroscience Meeting (San Diego).

Crepel, F., and Daniel, H. (2007). Developmental changes in agonist-induced retrograde signaling at parallel fiber-Purkinje cell synapses: role of calcium-induced calcium release. J Neurophysiol 98, 25502565.

Crunelli, V., Cope, D.W., and Hughes, S.W. (2006). Thalamic T-type Ca2+ channels and NREM sleep. Cell Calcium 40, 175-190.

Denk, W., Sugimori, M., and Llinas, R. (1995). Two types of calcium response limited to single spines in cerebellar Purkinje cells. Proc Natl Acad Sci U S A 92, 8279-8282.

Dove, L.S., Abbott, L.C., and Griffith, W.H. (1998). Whole-cell and single-channel analysis of P-type calcium currents in cerebellar Purkinje cells of leaner mutant mice. J Neurosci 18, 7687-7699.

Duguid, I.C., Pankratov, Y., Moss, G.W., and Smart, T.G. (2007). Somatodendritic release of glutamate regulates synaptic inhibition in cerebellar Purkinje cells via autocrine mGluR1 activation. J Neurosci 27, 12464-12474.

Dzubay, J.A., and Otis, T.S. (2002). Climbing fiber activation of metabotropic glutamate receptors on cerebellar purkinje neurons. Neuron 36, 1159-1167.

Edgerton, J.R., and Reinhart, P.H. (2003). Distinct contributions of small and large conductance Ca2+activated K+ channels to rat Purkinje neuron function. J Physiol 548, 53-69.

Egger, V., Svoboda, K., and Mainen, Z.F. (2003). Mechanisms of lateral inhibition in the olfactory bulb: efficiency and modulation of spike-evoked calcium influx into granule cells. J Neurosci 23, 7551-7558.

Egger, V., Svoboda, K., and Mainen, Z.F. (2005). Dendrodendritic synaptic signals in olfactory bulb granule cells: local spine boost and global low-threshold spike. J Neurosci 25, 3521-3530.

Erickson, M.A., Haburcak, M., Smukler, L., and Dunlap, K. (2007). Altered functional expression of Purkinje cell calcium channels precedes motor dysfunction in tottering mice. Neuroscience 150, 547555.

Finch, E.A., and Augustine, G.J. (1998). Local calcium signalling by inositol-1,4,5-trisphosphate in Purkinje cell dendrites. Nature 396, 753-756.

Fox, A.P., Nowycky, M.C., and Tsien, R.W. (1987). Kinetic and pharmacological properties distinguishing three types of calcium currents in chick sensory neurones. J Physiol 394, 149-172.

Gamper, N., and Shapiro, M.S. (2007). Regulation of ion transport proteins by membrane phosphoinositides. Nat Rev Neurosci 8, 921-934.

Hartmann, J., and Konnerth, A. (2005). Determinants of postsynaptic Ca2+ signaling in Purkinje neurons. Cell Calcium 37, 459-466.

Hashimoto, K., and Kano, M. (2005). Postnatal development and synapse elimination of climbing fiber to Purkinje cell projection in the cerebellum. Neuroscience research 53, 221-228.

Hughes, S.W., Cope, D.W., Blethyn, K.L., and Crunelli, V. (2002). Cellular mechanisms of the slow (<1 Hz ) oscillation in thalamocortical neurons in vitro. Neuron 33, 947-958.

Huguenard, J.R., and Prince, D.A. (1992). A novel T-type current underlies prolonged $\mathrm{Ca}(2+$ )-dependent burst firing in GABAergic neurons of rat thalamic reticular nucleus. J Neurosci 12, 3804-3817.

Isope, P., and Murphy, T.H. (2005). Low threshold calcium currents in rat cerebellar Purkinje cell dendritic spines are mediated by T-type calcium channels. J Physiol 562, 257-269.

Joksovic, P.M., Bayliss, D.A., and Todorovic, S.M. (2005). Different kinetic properties of two T-type $\mathrm{Ca} 2+$ currents of rat reticular thalamic neurones and their modulation by enflurane. J Physiol 566, 125142.

Joksovic, P.M., Nelson, M.T., Jevtovic-Todorovic, V., Patel, M.K., Perez-Reyes, E., Campbell, K.P., Chen, C.C., and Todorovic, S.M. (2006). CaV3.2 is the major molecular substrate for redox regulation of T-type Ca2+ channels in the rat and mouse thalamus. J Physiol 574, 415-430.

Jones, S.V. (1993). Muscarinic receptor subtypes: modulation of ion channels. Life Sci 52, 457-464.

Kawano, T., Zhao, P., Floreani, C.V., Nakajima, Y., Kozasa, T., and Nakajima, S. (2007). Interaction of Galphaq and Kir3, G protein-coupled inwardly rectifying potassium channels. Mol Pharmacol 71, 11791184.

Khosravani, H., and Zamponi, G.W. (2006). Voltage-gated calcium channels and idiopathic generalized epilepsies. Physiol Rev 86, 941-966.

Kim, D., Song, I., Keum, S., Lee, T., Jeong, M.J., Kim, S.S., McEnery, M.W., and Shin, H.S. (2001). Lack of the burst firing of thalamocortical relay neurons and resistance to absence seizures in mice lacking alpha(1G) T-type $\mathrm{Ca}(2+$ ) channels. Neuron 31, 35-45.

Kim, J.A., Park, J.Y., Kang, H.W., Huh, S.U., Jeong, S.W., and Lee, J.H. (2006). Augmentation of Cav3.2 T-type calcium channel activity by cAMP-dependent protein kinase A. J Pharmacol Exp Ther 318, 230-237.

Kim, S.J., Kim, Y.S., Yuan, J.P., Petralia, R.S., Worley, P.F., and Linden, D.J. (2003). Activation of the TRPC1 cation channel by metabotropic glutamate receptor mGluR1. Nature 426, 285-291.

Kitano, J., Nishida, M., Itsukaichi, Y., Minami, I., Ogawa, M., Hirano, T., Mori, Y., and Nakanishi, S. (2003). Direct interaction and functional coupling between metabotropic glutamate receptor subtype 1 and voltage-sensitive Cav2.1 Ca2+ channel. J Biol Chem 278, 25101-25108.

Knopfel, T., and Grandes, P. (2002). Metabotropic glutamate receptors in the cerebellum with a focus on their function in Purkinje cells. Cerebellum 1, 19-26.

Koike-Tani, M., Collins, J.M., Kawano, T., Zhao, P., Zhao, Q., Kozasa, T., Nakajima, S., and Nakajima, Y. (2005). Signal transduction pathway for the substance P-induced inhibition of rat Kir3 (GIRK) channel. J Physiol 564, 489-500.

Kulik, A., Nakadate, K., Hagiwara, A., Fukazawa, Y., Lujan, R., Saito, H., Suzuki, N., Futatsugi, A., Mikoshiba, K., Frotscher, M., and Shigemoto, R. (2004). Immunocytochemical localization of the alpha 1A subunit of the P/Q-type calcium channel in the rat cerebellum. Eur J Neurosci 19, 2169-2178.

Lee, J.H., Gomora, J.C., Cribbs, L.L., and Perez-Reyes, E. (1999). Nickel block of three cloned T-type calcium channels: low concentrations selectively block alpha1H. Biophys J 77, 3034-3042.

Levey, A.I., Edmunds, S.M., Heilman, C.J., Desmond, T.J., and Frey, K.A. (1994). Localization of muscarinic m 3 receptor protein and M3 receptor binding in rat brain. Neuroscience 63, 207-221.

Lopez-Bendito, G., Shigemoto, R., Lujan, R., and Juiz, J.M. (2001). Developmental changes in the localisation of the mGluR1alpha subtype of metabotropic glutamate receptors in Purkinje cells. Neuroscience 105, 413-429.

McCormick, D.A. (1992). Cellular mechanisms underlying cholinergic and noradrenergic modulation of neuronal firing mode in the cat and guinea pig dorsal lateral geniculate nucleus. J Neurosci 12, 278-289.

McKay, B.E., McRory, J.E., Molineux, M.L., Hamid, J., Snutch, T.P., Zamponi, G.W., and Turner, R.W. (2006). $\mathrm{Ca}(\mathrm{V}) 3$ T-type calcium channel isoforms differentially distribute to somatic and dendritic compartments in rat central neurons. Eur J Neurosci 24, 2581-2594.

McRory, J.E., Santi, C.M., Hamming, K.S., Mezeyova, J., Sutton, K.G., Baillie, D.L., Stea, A., and Snutch, T.P. (2001). Molecular and functional characterization of a family of rat brain T-type calcium channels. J Biol Chem 276, 3999-4011.

Mintz, I.M., Adams, M.E., and Bean, B.P. (1992). P-type calcium channels in rat central and peripheral neurons. Neuron 9, 85-95.

Nahm, S.S., Jung, K.Y., Enger, M.K., Griffith, W.H., and Abbott, L.C. (2005). Differential expression of T-type calcium channels in P/Q-type calcium channel mutant mice with ataxia and absence epilepsy. Journal of neurobiology 62, 352-360.

Nakamura, M., Sato, K., Fukaya, M., Araishi, K., Aiba, A., Kano, M., and Watanabe, M. (2004). Signaling complex formation of phospholipase Cbeta4 with metabotropic glutamate receptor type 1alpha and 1,4,5-trisphosphate receptor at the perisynapse and endoplasmic reticulum in the mouse brain. Eur J Neurosci 20, 2929-2944.

Nimchinsky, E.A., Sabatini, B.L., and Svoboda, K. (2002). Structure and function of dendritic spines. Annu Rev Physiol 64, 313-353.

Pellegrini, A., Dossi, R.C., Dal Pos, F., Ermani, M., Zanotto, L., and Testa, G. (1989). Ethosuximide alters intrathalamic and thalamocortical synchronizing mechanisms: a possible explanation of its antiabsence effect. Brain Res 497, 344-360.

Plummer, K.L., Manning, K.A., Levey, A.I., Rees, H.D., and Uhlrich, D.J. (1999). Muscarinic receptor subtypes in the lateral geniculate nucleus: a light and electron microscopic analysis. J Comp Neurol 404, 408-425.

Randall, A.D., and Tsien, R.W. (1997). Contrasting biophysical and pharmacological properties of Ttype and R-type calcium channels. Neuropharmacology 36, 879-893.

Robbins, J., Marsh, S.J., and Brown, D.A. (2006). Probing the regulation of M (Kv7) potassium channels in intact neurons with membrane-targeted peptides. J Neurosci 26, 7950-7961.

Sandona, D., Scolari, A., Mikoshiba, K., and Volpe, P. (2003). Subcellular distribution of Homer 1b/c in relation to endoplasmic reticulum and plasma membrane proteins in Purkinje neurons. Neurochemical research 28, 1151-1158.

Sdrulla, A.D., and Linden, D.J. (2007). Double dissociation between long-term depression and dendritic spine morphology in cerebellar Purkinje cells. Nat Neurosci 10, 546-548.

Shin, J.H., Kim, Y.S., and Linden, D.J. (2008). Dendritic glutamate release produces autocrine activation of mGluR1 in cerebellar Purkinje cells. Proc Natl Acad Sci U S A 105, 746-750.

Simen, A.A., Lee, C.C., Simen, B.B., Bindokas, V.P., and Miller, R.J. (2001). The C terminus of the Ca channel alpha1B subunit mediates selective inhibition by G-protein-coupled receptors. J Neurosci 21, 7587-7597.

Spacey, S.D., Hildebrand, M.E., Materek, L.A., Bird, T.D., and Snutch, T.P. (2004). Functional implications of a novel EA2 mutation in the P/Q-type calcium channel. Ann Neurol 56, 213-220.

Steriade, M. (2004). Acetylcholine systems and rhythmic activities during the waking--sleep cycle. Progress in brain research 145, 179-196.

Sterne-Marr, R., Tesmer, J.J., Day, P.W., Stracquatanio, R.P., Cilente, J.A., O'Connor, K.E., Pronin, A.N., Benovic, J.L., and Wedegaertner, P.B. (2003). G protein-coupled receptor Kinase 2/G alpha q/11 interaction. A novel surface on a regulator of G protein signaling homology domain for binding G alpha subunits. J Biol Chem 278, 6050-6058.

Suh, B.C., and Hille, B. (2005). Regulation of ion channels by phosphatidylinositol 4,5-bisphosphate. Curr Opin Neurobiol 15, 370-378.

Swensen, A.M., and Bean, B.P. (2003). Ionic mechanisms of burst firing in dissociated Purkinje neurons. J Neurosci 23, 9650-9663.

Tai, C., Kuzmiski, J.B., and MacVicar, B.A. (2006). Muscarinic enhancement of R-type calcium currents in hippocampal CA1 pyramidal neurons. J Neurosci 26, 6249-6258.

Takechi, H., Eilers, J., and Konnerth, A. (1998). A new class of synaptic response involving calcium release in dendritic spines. Nature 396, 757-760.

Talley, E.M., Cribbs, L.L., Lee, J.H., Daud, A., Perez-Reyes, E., and Bayliss, D.A. (1999). Differential distribution of three members of a gene family encoding low voltage-activated (T-type) calcium channels. J Neurosci 19, 1895-1911.

Tsay, D., and Yuste, R. (2004). On the electrical function of dendritic spines. Trends in neurosciences 27, 77-83.

Tu, J.C., Xiao, B., Yuan, J.P., Lanahan, A.A., Leoffert, K., Li, M., Linden, D.J., and Worley, P.F. (1998). Homer binds a novel proline-rich motif and links group 1 metabotropic glutamate receptors with IP3 receptors. Neuron 21, 717-726.

Usowicz, M.M., Sugimori, M., Cherksey, B., and Llinas, R. (1992). P-type calcium channels in the somata and dendrites of adult cerebellar Purkinje cells. Neuron 9, 1185-1199.

Wang, S.S., Denk, W., and Hausser, M. (2000). Coincidence detection in single dendritic spines mediated by calcium release. Nat Neurosci 3, 1266-1273.

Watanabe, S., Takagi, H., Miyasho, T., Inoue, M., Kirino, Y., Kudo, Y., and Miyakawa, H. (1998). Differential roles of two types of voltage-gated $\mathrm{Ca} 2+$ channels in the dendrites of rat cerebellar Purkinje neurons. Brain Res 791, 43-55.

Welsby, P.J., Wang, H., Wolfe, J.T., Colbran, R.J., Johnson, M.L., and Barrett, P.Q. (2003). A mechanism for the direct regulation of T-type calcium channels by Ca2+/calmodulin-dependent kinase II. J Neurosci 23, 10116-10121.

Williams, S.R., Christensen, S.R., Stuart, G.J., and Hausser, M. (2002). Membrane potential bistability is controlled by the hyperpolarization-activated current $\mathrm{I}(\mathrm{H})$ in rat cerebellar Purkinje neurons in vitro. J Physiol 539, 469-483.

Williams, S.R., Toth, T.I., Turner, J.P., Hughes, S.W., and Crunelli, V. (1997). The 'window' component of the low threshold $\mathrm{Ca} 2+$ current produces input signal amplification and bistability in cat and rat thalamocortical neurones. J Physiol 505 ( Pt 3), 689-705.

Wolfe, J.T., Wang, H., Howard, J., Garrison, J.C., and Barrett, P.Q. (2003). T-type calcium channel regulation by specific G-protein betagamma subunits. Nature 424, 209-2 13 .

Womack, M., and Khodakhah, K. (2002). Active contribution of dendrites to the tonic and trimodal patterns of activity in cerebellar Purkinje neurons. J Neurosci 22, 10603-10612.

Womack, M.D., Chevez, C., and Khodakhah, K. (2004). Calcium-activated potassium channels are selectively coupled to P/Q-type calcium channels in cerebellar Purkinje neurons. J Neurosci 24, 88188822.

Womack, M.D., and Khodakhah, K. (2004). Dendritic control of spontaneous bursting in cerebellar Purkinje cells. J Neurosci 24, 3511-3521.

Yasuda, R., Sabatini, B.L., and Svoboda, K. (2003). Plasticity of calcium channels in dendritic spines. Nat Neurosci 6, 948-955.

Yuan, J.P., Kiselyov, K., Shin, D.M., Chen, J., Shcheynikov, N., Kang, S.H., Dehoff, M.H., Schwarz, M.K., Seeburg, P.H., Muallem, S., and Worley, P.F. (2003). Homer binds TRPC family channels and is required for gating of TRPC1 by IP3 receptors. Cell 114, 777-789.

Zamponi, G.W., Bourinet, E., Nelson, D., Nargeot, J., and Snutch, T.P. (1997). Crosstalk between G proteins and protein kinase C mediated by the calcium channel alpha1 subunit. Nature 385, 442-446.

Zamponi, G.W., Bourinet, E., and Snutch, T.P. (1996). Nickel block of a family of neuronal calcium channels: subtype- and subunit-dependent action at multiple sites. J Membr Biol 151, 77-90.

# APPENDIX 1: CONTRIBUTIONS OF T-TYPE CALCIUM CHANNELS TO THE PATHOPHYSIOLOGY OF PAIN SIGNALING* 

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Pain

# Contributions of T-type calcium channels to the pathophysiology of pain signaling 

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Among the family of voltage-gated calcium channels, low threshold T-type channels have only recently joined their high threshold N-type channel brother in being generally accepted as molecular players within pain signaling. The ability of T-type channels to modulate neuronal excitability at low thresholds has implications concerning their participation in pain neurotransmission at multiple levels including peripheral nociceptors, the spinal cord and the brain. Consensus is rapidly developing that in the periphery and spinal cord the Cav3.2 T-type calcium channel isoform selectively plays a pronociceptive role and therefore represents an attractive target for future therapeutic strategies.

## Introduction

The unpleasant sensory and emotional experiences known as pain are caused by electrical signals that are relayed through well-defined neuronal pathways to specific areas of the brain (for reviews, see [1,2]). The encoding and processing of these potential or actual tissue-damaging (noxious) events in the nervous system is referred to as nociception. Noxious stimuli activate sensor receptors (e.g. sensitive to heat, pressure, acid, inflammation) to generate sensor potentials in peripheral pain-sensing free nerve endings of the nociceptors. Upon reaching sufficient amplitude, the

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potentials trigger action potentials (APs) at the soma of nociceptors (dorsal root ganglion, DRG), which then conduct down axons to the superficial laminae located in the dorsal horn of the spinal cord. Subsequent activation of dorsal horn spinal neurons by neurotransmitter release (primarily glutamate) from the nociceptors causes the generation of APs that propagate to the thalamus, mainly through the spinothalamic tract (STT) pathway (Fig. 1). Specific thalamic nuclei innervated by the STT relay the nociceptive signals to areas of the brain responsible for the sensory discriminatory (lateral thalamocortical system) and affective (medial thalamocortical system) aspects of the painful stimuli. Generally, both tissue inflammation (inflammatory pain) and damage to neurons (neuropathic pain) can result in pathophysiological pain wherein nociceptors fire spontaneously or are hyper-responsive to either innocuous (allodynic) or noxious (hyperalgesic) stimuli. Although low voltage-activated (LVA) T-type voltage-gated calcium (Ca) channels are well known to be expressed in pain pathway components [3,4], a lack of specific antagonists and molecular genetic tools delayed the elucidation of their specific contributions in pain signaling. Most recently, the defining of three distinct genes encoding for T-type Ca channels has lead to new tools utilized to uncover their involvement in nociceptive processes.

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Figure I. Involvement of T-type calcium channels in the nociceptive system. Scheme summarizing the locations of T-type Ca channels and the pathways that they contribute towards pain signaling. Abbreviations: VPL, ventroposterolateral nucleus; DRG, dorsal root ganglion. Adapted from reference [2].

## A brief history of T-type calcium channels

Twenty-five years ago, Llinas and Yarom used current clamp intracellular recordings on inferior olive brain slices to identify low-threshold Ca spikes linked to both a prolonged depolarization after the initial $\mathrm{Na}^{+}$spike of the AP (called the after-depolarizing potential, ADP) and to rebound burst firing after membrane hyperpolarization [5,6]. Shortly thereafter, Carbone and Lux used whole-cell and single channel patch-clamp recordings in an in vitro preparation of chick and rat sensory neurons to define the biophysical properties of LVA Ca channels [4]. Subsequent studies in isolated DRGs demonstrated that LVA Ca currents (now called T-type) could be distinguished from high voltage-activated (HVA) Ca currents by their faster kinetics of activation and inactivation, slower deactivation kinetics, more hyperpolarized voltagedependence of activation and inactivation, and smaller sin-gle-channel conductance [7-9]. Overall, these distinct biophysical properties enable T-type Ca channels to regulate subthreshold excitability including mediating bursting behaviors and firing rates (reviewed in [3]). The observed heterogeneity in the biophysical properties of native T-type Ca currents found in various types of neurons can largely be explained by the existence of three distinct genes that encode for Ca channel $\alpha_{1}$ subunit proteins with LVA biophysical
properties: Cav3.1 ( $\alpha_{1 \mathrm{G}}$ ), $\operatorname{Cav} 3.2\left(\alpha_{1 \mathrm{H}}\right)$ and $\operatorname{Cav} 3.3\left(\alpha_{11}\right)$ (reviewed in [10]). The three T-type $\alpha_{1}$ subunits are differentially expressed at the cellular and subcellular levels throughout the nervous system and there also exist several alternatively spliced variants that generate further T-type biophysical diversity [11-15]. While high affinity, subtype specific pharmacological agents that can distinguish between the Cav T-type isoforms have yet to be developed, the channels exhibit several unique properties (e.g., Cav3.2 has an $\sim$ tenfold higher sensitivity to $\mathrm{Ni}^{2+}$ blockade; Cav3.3 has proportionally slower activation and inactivation kinetics and faster deactivation kinetics, Cav3.1 has a greater permeability to $\mathrm{Ca}^{2+}$ than $\left.\mathrm{Ba}^{2+}[10,16]\right)$ that permit at least partial distinctions to be made.

## T-type calcium channels in peripheral nociceptors

 Cav3.2 is the major T-type channel isoform expressed in peripheral nociceptorsPeripheral sensory neurons are classically divided into functional groups based on axonal conduction velocity, which itself is positively correlated with cell soma size [17]. Approximate classifications based upon DRG diameter can be made: small diameter DRGs ( $15-30 \mu \mathrm{~m}$ ) are slowly conducting unmyelinated C sensory fibers, medium diameter DRGs
(31-40 $\mu \mathrm{m}$ ) are thin, myelinated A $\delta$ fibers, and large diameter DRGs $(45-51 \mu \mathrm{~m})$ are large myelinated $A \alpha$ and $A \beta$ sensory fibers. Peripheral nociceptors are known to be composed of C- and A $\delta$-sensory fibers [18]. Most small-size DRGs are sensitive to capsaicin and exhibit long duration APs, both hallmarks of nociceptors, whereas a majority of medium-sized DRG neurons do not respond to capsaicin, raising the question of which medium-sized DRGs are in fact nociceptors [19]. Although somewhat variable from cell to cell, small DRGs generally express lower levels of whole-cell T-type currents (up to 250 pA ) compared to some medium-sized DRGs with very large T-type currents ( $>4000 \mathrm{pA}$; and often designated as D-hair mechanoreceptors) and little to no observable T-type conductances in large DRGs [19-21]. Todorovic and co-workers [22] have recently identified a novel subset of small DRGs (26-31 $\mu \mathrm{m}$ ) named 'T-Rich' that express both capsaicin receptors and the isolectin $\beta_{4}\left(\mathrm{IB}_{4}\right)$ nociceptor marker and possess a high density of T-type currents (up to 3000 pA ) with virtually no HVA Ca currents.

Several lines of evidence demonstrate that Cav3.2 is the predominant T-type Ca channel isoform expressed in peripheral nociceptors. In situ hybridization and quantitative RTPCR for all three T-type channel isoforms reveals that Cav3.2 is transcribed at significantly higher levels (five to tenfold) compared to Cav3.1 and Cav3.3 in small and medium DRGs [15,23]. Electrophysiologically, T-type currents in small and medium DRGs also exhibit fast inactivation kinetics and potent block by $\mathrm{Ni}^{2+}$, properties that are characteristic of Cav3.2 channels [8,23]. Finally, conclusive evidence arises from experiments examining Cav3.2 gene knock-out (KO) mice, wherein T-type whole-cell currents are functionally absent in the small DRGs [24].

T-type channels are linked to excitability in peripheral nociceptors Through a combination of biophysical, pharmacological and ion substitution experiments, White et al. [25] have shown a role for T-type currents in switching from tonic to phasic firing in sensory neurons. The authors find that in 30-50 $\mu \mathrm{m}$ medium to large DRGs, T-type currents contribute to the ADP which is occasionally crowned with repetitive bursts. Recent studies with action potential command waveforms suggest that in small DRGs T-type Ca channels have a minor role in directly contributing to Ca ion entry during the ADP, whereas tetrodotoxin-resistant Na channels and HVA Ca channels carry most of the inward current during the ADP shoulder [26]. By contrast, the T-Rich subset of nociceptors (26-31 $\mu \mathrm{m}$ DRGs) have a high density of T-type current that when robustly enhanced by the endogenous amino acid, l-cysteine, results in a lower depolarization threshold for excitability and a concomitant increase in the probability of burst firings containing APs and ADPs. Computer simulations demonstrate that the increase in nociceptor excitability is due to a hyperpolarizing shift in the voltage-dependence of T-type
activation, whereas loss-of-function experiments with $100 \mu \mathrm{M} \mathrm{Ni}^{2+}$ (blocking Cav3.2) show that loss of T-type current causes a significant decrease in the amplitude of the ADP and an increase in the threshold for AP firing [22].

Heppenstall and Lewin [27] have recently argued that it is unclear in vivo whether T-rich sensory cells are nociceptors or rather are low-threshold mechanoreceptors: (1) the T-rich DRGs have short-duration APs which are 'in vivo invariably a unique property of low-threshold mechanoreceptors' and (2) the T-rich experiments examined the cell bodies of sensory neurons grown in culture, where some ionic properties can be lost or altered. We would counter that in a detailed characterization by Cardenas et al., a prevalent group of small DRGs ( $25 \mu \mathrm{~m}$ mean diameter) had both short duration APs and were highly capsaicin sensitive and thus, were most likely nociceptors and distinct from mechanoreceptors [19]. Additionally, Nelson et al. [22] have identified small diameter DRGs with similar T-Rich biophysical and pharmacological properties in whole-cell recordings of an intact DRG preparation. Overall, combining the aspects of DRG size, positive nociceptive markers, and a hyperpolarizing response to serotonin application (which is characteristic of C - and $\mathrm{A} \delta$ sensory fibers; [22]) makes it more likely that T-Rich DRGs are in fact a subset of nociceptors rather than low-threshold mechanoreceptors.

T-type currents contribute to both acute peripheral nociception and neuropathic pain
Physiologically, T-type Ca channels have been linked to peripheral nociception in studies showing that the endogenous reducing agent l-cysteine both enhances T-type currents in small DRGs in vitro and also causes thermal and mechanical hyperalgesia in vivo. Conversely, an endogenous inhibitor of T-type currents, allopregnanolone, promotes thermal and mechanical analgesia in vivo $[28,29]$. This evidence for the pronociceptive role of T-type currents in peripheral nociceptors is strengthened by observations that the amounts of L cysteine that increase T-type currents in vitro and that are hyperalgesic in vivo are within physiologically-relevant concentration ranges [29]. A variety of other pharmacological agents block T-type Ca channels with varying degrees of affinity and specificity including clinical antiepileptics (ethosuximide, phenytoin, zonisamide), antipsychotics (penfluridol, pimozide) and antiarrthymics (bepridil, mibefradil, efondipine), as well as dietary agents ( $\omega$-fatty acids) and endogenous messengers (endocannabinoids). Extensive studies examining these weakly-selective agents in acute and neuropathic pain animal models as well as human pain conditions are highly suggestive that modulation of T-type Ca channel activity is a valid approach to the broad treatment of pain conditions (reviewed in [30]).

A recent study by Bourinet et al. was the first to move past pharmacological correlations and to directly demonstrate a
role for the Cav3.2 T-type channel isoform in peripheral nociceptors [23]. Intrathecal administration of Cav3.2-specific, but not Cav3.1- or Cav3.3-specific, antisense oligonucleotides results in antiallodynic and analgesic effects in rat models of both acute thermal and mechanical pain. The local injection of Cav3.2 antisense oligonucleotides also results in the significant and long-term attenuation of surgicallyinduced neuropathic hypersensitivity [23]. The robust antinociceptive effects mediated by reducing Cav3.2 T-type channel expression are similar or greater in amplitude but have greater duration of action than reference analgesics such as morphine and clomipramine. Control experiments show that Cav3.2 antisense injection results in a dramatic reduction in expression of both Cav3.2 mRNA and protein in DRGs of the targeted SC regions, as well as a $75 \%$ and $92 \%$ decrease in T-type whole-cell currents in small and medium DRGs, respectively [23].

Most recently, Choi et al. have characterized the pain susceptibility of Cav3.2 gene KO mice that exhibit normal locomotion, motor coordination and anxiety levels [31]. The authors find attenuated responses to mechanical, thermal, and chemical cutaneous pain stimuli as well as chemical visceral pain stimuli in the Cav3.2 gene KO mice thus providing further direct evidence for a pronociceptive role of Cav3.2 in the periphery. In contrast to Cav3.2 antisense studies examining the rat chronic constrictive injury (CCI) model of neuropathic pain [23], Cav3.2 gene KO mice that had spinal-nerve ligation (SNL)-induced neuropathic pain showed no significant differences in behavioral responses when compared to wild-type mice with the same treatment.

In the CCI rat model of neuropathic pain, local injection of inhibitors of T-type channels, including mibefradil, oxidizing agents, neuroactive steroids and ethosuximide all result in a reduction of CCI-induced hyperalgesia, and in some cases abolish the progression of neuropathic pain [32-34]. The reduction in the ability of reducing agents such as L -cysteine to induce hyperalgesia and the robust analgesia induced by oxidizing agents such as 5,5'-dithio-bis-(2-nitrobenzoic acid) in CCI rats has lead to the hypothesis that the metabolic stress induced by neuronal injury causes a reduced peripheral cellular environment wherein T-type channels are in a more sensitized state, contributing to hypersensitivity of nociception $[33,35]$. In support of this notion, local and intraperitoneal administration, but not intrathecal administration, of mibefradil relieves CCI-induced tactile and thermal hypersensitivity, suggesting a peripheral site of action [34].

There have been conflicting reports that the levels of T-type channel expression in DRGs either does not change [36] or decreases [37] in neuropathic animal models compared to wild-type animals. These studies examined acutely dissociated medium-sized DRGs (between 33 and $50 \mu \mathrm{~m}$ ), the majority of which are likely not nociceptors, thus additional studies are required examining T-type current density in
intact peripheral nociceptors (mostly small DRGs) in animal models of neuropathic pain. This highlights one of the biggest limitations of most studies of T-type channels in peripheral nociceptors: almost all electrophysiological recordings are from acutely dissociated or cultured DRGs that contain only the nociceptor soma. T-type channels are well known to be highly expressed in dendrites throughout the nervous system [38-41] and in peripheral nociceptors they may play significant roles defining the excitability of nerve endings, although this has largely been unexplored. It is likely that high resolution Ca imaging combined with subtype specific T-type channel antagonists and antibodies will be required to investigate these issues.

## T-type calcium channels in the spinal cord

Under conditions of severe or persistent nociceptive signaling, C-fibers can fire repetitively and induce a progressive increase in the response of the dorsal horn spinal projection neurons (PNs) that express neurokinin-1 receptors $\left(\mathrm{NK}_{1}\right)$ and that have ascending connections to the brain. This frequency-dependent form of synaptic potentiation between C-fibers and superficial laminae spinal neurons is known as wind-up and shares many properties with both central sensitization and the spinal cord-mediated hyperalgesia observed after peripheral nerve damage [42]. Similar to that for peripheral nociceptors, in situ hybridization reveals that Cav3.2 is the primary T-type channel isoform expressed in the superficial layers of the dorsal horn of the spinal cord [15]. Application of $100 \mu \mathrm{M} \mathrm{Ni}^{2+}$ blocks a significant portion of voltage-gated Ca transients in lamina I neurons of the dorsal horn, suggestive of functional T-type currents in these cells being most likely encoded by the Cav3.2 channel [43].

In work directly demonstrating a nociceptive role for spinal T-type channels, Ikeda et al. have shown that high frequency stimulation of C-fibers causes release of substance P that activates $\mathrm{NK}_{1}$ receptors on the lamina I PNs, leading to a signal transduction pathway that facilitates NMDA receptors and increases cytosolic Ca levels (Fig. 2). The synergistic activation of NMDA receptors and T-type currents increases intracellular Ca levels and potentiates postsynaptic responses [44]. This Ca-dependent wind-up phenomenon appears to only occur in PNs wherein the presence of T-type currents allows for both broadening of the action potential and a more negative threshold for firing.

Further support for a role of T-type channels in spinal pain signaling comes from in vivo electrophysiological recording experiments in sham-operated and CCI rats in which intrathecal injection of a weakly selective T-type antagonist, ethosuximide, dose-dependently reduces the responses of dorsal horn neurons to innocuous and noxious electrical, mechanical, and thermal stimuli [45]. Also, $\mu$-opioid receptors that morphine acts upon are located in lamina I/II of the


Figure 2. Potentiation of spinal projection neuron responses requires T-type calcium channels. High frequency stimulation of nociceptive C -fibers results in the release of glutamate and substance $P$, which directly activate ligand-gated ionotropic (AMPAR and NMDAR) and metabotropic ( $\mathrm{NK}_{1}$ ) receptors, respectively. This leads to activation of signal transduction pathways that result in cell depolarization and increases in cytosolic $\mathrm{Ca}^{2+}$. All listed elements are necessary for the potentiation of excitatory post synaptic currents (EPSCs): blockers of NK, (L-703,606), intracellular Ca ${ }^{2+}$ (BAPTA), PLC (U73I22), IP R (2-APB), NMDAR (D-AP5), and T-type Ca channels ( $100 \mu \mathrm{MNi}^{2+}$ ) all abolish the induction of LTP. Other known intracellular elements that contribute to PN LTP and speculative linking molecules (such as CAMKII which is activated by $\mathrm{Ca}^{2+}$ and activates Cav3.2 T-type channels [58]) are not included in the figure. The figure is based on results and interpretations from reference [44].
dorsal horn and intrathecal injection of the T-type antagonist, mibefradil, potentiates both morphine- and [D-Ala(2), N mePhe(4), Gly-ol(5)] enkephalin (DAMGO)-induced antinociception [46].

## T-Type calcium channels in the thalamus

Differential expression and function of T-type channel isoforms in thalamic neurons
Two of the main cell types in the thalamus are relay thalamocortical (TC) neurons, which relay signals from the periphery to various regions of the cortex, and the GABAergic reticular thalamic neurons (nRT), which hyperpolarize TC neurons to induce rebound burst firing. In situ hybridization reveals that Cav3.1 is the most abundant T-type isoform in TCs whereas Cav3.2 and Cav3.3 mRNAs are more highly expressed in nRT cells [15]. Consistent with these findings, whole-cell T-type currents in TC cells are completely abolished in Cav3.1 gene KO mice, suggesting that Cav3.1 underlies functional T-type currents in these cells [47]. Electrophysiological recordings of nRT cells reveal two distinct populations of T-type currents with properties similar to that for the Cav3.2 and Cav3.3 isoforms [39,48]. The observation that there remains a significant T-type current in the nRT
cells of Cav3.2 gene KO mice is also suggestive that both isoforms are normally present in these cells [49].
T-type currents appear to play an essential role in the reciprocal firing interactions between TC and nRT cells that both initiate oscillating rebound bursting patterns and the spindle waves that underlie slow-wave sleep [50]. Indeed, altering T-type currents in these cells abolishes reboundburst firing and the proper timing of AP firing and disrupts sleep gating [47,51]. Over-activity of T-type currents in TC and nRT cells is also associated with pathophysiological spike-and-wave discharges that underlie absence epilepsy [47,52]. A linkage between the thalamus and higher level pain signaling is demonstrated in findings that lesions in the lateral spinothalamic system can result in neurogenic pain, whereas medial thalamotomies can relieve neurogenic pain [53]. Of particular relevance, electrophysiological recordings can be made from the lateral thalamic nuclei in awake patients with chronic neurogenic pain. TC cells from these patients display abnormal low-threshold spikes and burst firing in frequency ranges ( $3-5 \mathrm{~Hz}$ ) which are normally associated with slow wave sleep and not wakefulness, potentially implicating involvement of T-type Ca channels [50,53].

## Thalamic Cav3.I currents are antinociceptive in visceral pain responses

As described above, in both the spinal cord and periphery, T-type Ca channels (mainly the Cav3.2 subtype) are implicated in pronociceptive mechanisms of somatic pain. By contrast, a recent study has implicated a novel antinociceptive role of thalamic Cav3.1 T-type channels in visceral pain mechanisms. Of note, Cav3.1 gene KO mice exhibit normal responses to thermal and mechanical somatic pain, as well as hyperalgesia to cutaneous pain similar to that for wild-type mice, providing supporting evidence that Cav3.2 is the major T-type isoform involved in SC and peripheral pain pathways [54]. However, in Cav3.1 KO animals the induction of visceral pain by the intraperitoneal injection of either acetic acid or $\mathrm{MgSO}_{4}$ results in significantly enhanced writhing responses. In phenocopy experiments with wild-type mice, intraperitoneal injection of mibefradil (which does not effectively cross the blood-brain-barrier) reduces visceral pain (likely through action on Cav3.2 channels [31]), whereas the focal injection of mibefradil into the ventroposterolateral (VPL) thalamus is pain-potentiating, suggesting that an antinoceptive-role of the Cav3.1 T-type channel in visceral pain is restricted to the thalamus.

In vivo membrane potential recordings of VPL neurons comparing Cav3.1 gene KO and wild type mice shows that while the firing rate of single AP spikes is similar to that for wild-type mice, the VPL neurons of the Cav3.1 gene KO mice have a significantly lower total firing frequency owing to a lack of burst firing. Although the onset of visceral pain in wild-type mice results in an initial surge of single spike APs followed by a gradual increase in burst spikes correlated with a decay in the elevated single spike firing rate, in Cav3.1 gene KO mice the initial increase of single AP spikes in response to visceral pain is sustained because burst-spike activity is not possible [54]. This suggests that the Cav3.1 T-type channels of the VPL are normally involved in sensory gating by inhibiting high frequency single spike visceral pain signals through the initiation of rebound burst firing. As intraperitoneal injections of acetic acid or $\mathrm{MgSO}_{4}$ are an artificial model of visceral pain, it will be interesting to determine whether a similar mechanism occurs under more physiological models of visceral luminal pain, such as colorectal distention or intracolonic perfusion of irritant substances [55]. Additionally, as noxious visceral pain signals reach the VPL through the dorsal column of the SC [56], the role of thalamic T-type Ca channels in pain signaling via classical nociceptive STT pathways remains to be explored.

## Conclusions

The differential expression of a biophysically diverse family of LVA T-type Ca channels contributes to the diversification of physiological functions in the nervous system, including that of the nociceptive pathways. Centrally expressed Cav3.1

T-type Ca channels appear to have an antinociceptive role in thalamic visceral pain responses and thus blockade of this channel by pharmacological agents that penetrate the CNS may prove to be pronociceptive. Contrastingly, evidence has emerged to support a pronociceptive role for Cav3.2 T-type Ca channels in modulating excitability and contributing to pain signaling within both peripheral nociceptors and spinal cord dorsal horn neurons. Together with the potential contributions of peripheral and spinal T-type Ca channels towards the central sensitization implicated in neuropathic pain mechanisms, the development of Cav3.2 selective blockers may provide a new avenue of clinical treatment concerning both acute and neuropathic conditions. Interestingly, the high voltage-activated N-type Ca channel (Cav2.2/ $\alpha_{1 B}$ ) localized to nociceptor presynaptic terminals in the spinal cord dorsal horn is the target of a marketed peptide for severe intractable pain (Prialt ${ }^{\mathrm{TM}}$ ), as well for several small organic molecules currently in development (reviewed in [57]). It is tempting to speculate that there might be an opportunity for novel pharmacological agents targeting both peripheral Cav3.2 T-type and spinal Cav2.2 N-type channels. Such mixed N- and T-type Ca channel blockers might provide a new and powerful strategy aimed at treating a broad spectrum of human pain conditions.

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## References

1 Basbaum, A.I. and Jessell, T.M. (2000) The perception of pain. In Principles of Neural Science (Kandel, E.R. et al. eds), pp. 472-491, McGraw-Hill
2 Schaible, H.G. and Richter, F. (2004) Pathophysiology of pain. Langenbecks Arch. Surg. 389, 237-243
3 Huguenard, J.R. (1996) Low-threshold calcium currents in central nervous system neurons. Annu. Rev. Physiol. 58, 329-348
4 Carbone, E. and Lux, H.D. (1984) A low voltage-activated, fully inactivating Ca channel in vertebrate sensory neurones. Nature 310, 501-502
5 Llinas, R. and Yarom, Y. (1981) Electrophysiology of mammalian inferior olivary neurones in vitro. Different types of voltage-dependent ionic conductances. J. Physiol. 315, 549-567
6 Llinas, R. and Yarom, Y. (1981) Properties and distribution of ionic conductances generating electroresponsiveness of mammalian inferior olivary neurones in vitro. J. Physiol. 315, 569-584
7 Fedulova, S.A. et al. (1985) Two types of calcium channels in the somatic membrane of new-born rat dorsal root ganglion neurones. J. Physiol. 359, 431-446

8 Fox, A.P. et al. (1987) Kinetic and pharmacological properties distinguishing three types of calcium currents in chick sensory neurones. J. Physiol. 394, 149-172
9 Bossu, J.L. et al. (1985) Depolarization elicits two distinct calcium currents in vertebrate sensory neurones. Pflugers Arch. 403, 360-368
10 Perez-Reyes, E. (2003) Molecular physiology of low-voltage-activated ttype calcium channels. Physiol. Rev. 83, 117-161
11 Molineux, M.L. et al. (2006) Specific T-type calcium channel isoforms are associated with distinct burst phenotypes in deep cerebellar nuclear neurons. Proc. Natl. Acad. Sci. U S A 103, 5555-5560
12 Mittman, S. et al. (1999) Structure and alternative splicing of the gene encoding alpha1I, a human brain T calcium channel alpha1 subunit. Neurosci. Lett. 269, 121-124
13 Monteil, A. et al. (2000) Molecular and functional properties of the human alpha(1G) subunit that forms T-type calcium channels. J. Biol. Chem. 275, 6090-6100
14 Monteil, A. et al. (2000) Specific properties of T-type calcium channels generated by the human alpha 1 I subunit. J. Biol. Chem. 275, 16530-16535
15 Talley, E.M. et al. (1999) Differential distribution of three members of a gene family encoding low voltage-activated (T-type) calcium channels. J. Neurosci. 19, 1895-1911
16 McRory, J.E. et al. (2001) Molecular and functional characterization of a family of rat brain T-type calcium channels. J. Biol. Chem. 276, 3999-4011
17 Harper, A.A. and Lawson, S.N. (1985) Conduction velocity is related to morphological cell type in rat dorsal root ganglion neurones. J. Physiol. 359, 31-46
18 Caterina, M.J. and Julius, D. (1999) Sense and specificity: a molecular identity for nociceptors. Curr. Opin. Neurobiol. 9, 525-530
19 Cardenas, C.G.et al. (1995) Variation in serotonergic inhibition of calcium channel currents in four types of rat sensory neurons differentiated by membrane properties. J. Neurophysiol. 74, 1870-1879
20 Scroggs, R.S. and Fox, A.P. (1992) Calcium current variation between acutely isolated adult rat dorsal root ganglion neurons of different size. J. Physiol. 445, 639-658

21 Shin, J.B. et al. (2003) A T-type calcium channel required for normal function of a mammalian mechanoreceptor. Nat. Neurosci. 6, 724-730
22 Nelson, M.T. et al. (2005) The endogenous redox agent l-cysteine induces T-type Ca2+ channel-dependent sensitization of a novel subpopulation of rat peripheral nociceptors. J. Neurosci. 25, 8766-8775
23 Bourinet, E. et al. (2005) Silencing of the Cav3.2 T-type calcium channel gene in sensory neurons demonstrates its major role in nociception. Embo J. 24, 315-324

24 Chen, C.C. et al. (2003) Abnormal coronary function in mice deficient in alpha1H T-type Ca2+ channels. Science 302, 1416-1418
25 White, G. et al. (1989) Transient low-threshold Ca2+ current triggers burst firing through an afterdepolarizing potential in an adult mammalian neuron. Proc. Natl. Acad. Sci. U S A 86, 6802-6806
26 Blair, N.T. and Bean, B.P. (2002) Roles of tetrodotoxin (TTX)-sensitive Na+ current, TTX-resistant $\mathrm{Na}+$ current, and $\mathrm{Ca} 2+$ current in the action potentials of nociceptive sensory neurons. J. Neurosci. 22, 10277-10290
27 Heppenstall, P.A. and Lewin, G.R. (2006) A role for T-type Ca(2+) channels in mechanosensation. Cell Calcium 40, 165-174
28 Pathirathna, S. et al. (2005) New evidence that both T-type calcium channels and GABAA channels are responsible for the potent peripheral analgesic effects of 5alpha-reduced neuroactive steroids. Pain 114, 429443
29 Todorovic, S.M. et al. (2001) Redox modulation of T-type calcium channels in rat peripheral nociceptors. Neuron 31, 75-85
30 Snutch, T.P. and David, L.S. (2006) T-type calcium channels: an emerging therapeutic target for the treatment of pain. Drug Dev. Res. 67, 404-415
31 Choi, S. et al. (2006) Attenuated pain responses in mice lacking Ca3.2 Ttype channels. Genes Brain Behav [Epub ahead of print] PMID: 16939637
32 Pathirathna, S. et al. (2005) 5alpha-reduced neuroactive steroids alleviate thermal and mechanical hyperalgesia in rats with neuropathic pain. Pain 117, 326-339
33 Todorovic, S.M. et al. (2004) Redox modulation of peripheral T-type Ca2+ channels in vivo: alteration of nerve injury-induced thermal hyperalgesia. Pain 109, 328-339

34 Dogrul, A. et al. (2003) Reversal of experimental neuropathic pain by Ttype calcium channel blockers. Pain 105, 159-168
35 Jevtovic-Todorovic, V. and Todorovic, S.M. (2006) The role of peripheral T-type calcium channels in pain transmission. Cell Calcium 40, 197-203
36 Baccei, M.L. and Kocsis, J.D. (2000) Voltage-gated calcium currents in axotomized adult rat cutaneous afferent neurons. J. Neurophysiol. 83, 2227-2238

37 McCallum, J.B. et al. (2003) Loss of T-type calcium current in sensory neurons of rats with neuropathic pain. Anesthesiology 98, 209-216
38 Magee, J. et al. (1998) Electrical and calcium signaling in dendrites of hippocampal pyramidal neurons. Annu. Rev. Physiol. 60, 327-346
39 Joksovic, P.M. et al. (2005) Different kinetic properties of two T-type Ca2+ currents of rat reticular thalamic neurones and their modulation by enflurane. J. Physiol. 566 (Pt 1), 125-142
40 McKay, B.E. et al. Cav3 T-type calcium channel isoforms differentially distribute to somatic and dendritic compartments in rat central neurons. Eur. J. Neurosci. (in press)
41 Isope, P. and Murphy, T.H. (2005) Low threshold calcium currents in rat cerebellar Purkinje cell dendritic spines are mediated by T-type calcium channels. J. Physiol. 562 (Pt 1), 257-269
42 Herrero, J.F. et al. (2000) Wind-up of spinal cord neurones and pain sensation: much ado about something? Prog. Neurobiol. 61, 169-203
43 Heinke, B. et al. (2004) Pre- and postsynaptic contributions of voltagedependent $\mathrm{Ca} 2+$ channels to nociceptive transmission in rat spinal lamina I neurons. Eur. J. Neurosci. 19, 103-111
44 Ikeda, H. et al. (2003) Synaptic plasticity in spinal lamina I projection neurons that mediate hyperalgesia. Science 299, 1237-1240
45 Matthews, E.A. and Dickenson, A.H. (2001) Effects of ethosuximide, a Ttype $\mathrm{Ca}(2+)$ channel blocker, on dorsal horn neuronal responses in rats. Eur. J. Pharmacol. 415, 141-149
46 Dogrul, A. et al. (2001) L-type and T-type calcium channel blockade potentiate the analgesic effects of morphine and selective mu opioid agonist, but not to selective delta and kappa agonist at the level of the spinal cord in mice. Pain 93, 61-68
47 Kim, D. et al. (2001) Lack of the burst firing of thalamocortical relay neurons and resistance to absence seizures in mice lacking alpha(1G) Ttype $\mathrm{Ca}(2+$ ) channels. Neuron 31, 35-45
48 Huguenard, J.R. and Prince, D.A. (1992) A novel T-type current underlies prolonged $\mathrm{Ca}(2+)$-dependent burst firing in GABAergic neurons of rat thalamic reticular nucleus. J. Neurosci. 12, 3804-3817
49 Joksovic, P.M. et al. (2006) CaV3.2 is the major molecular substrate for redox regulation of T-type $\mathrm{Ca} 2+$ channels in the rat and mouse thalamus. J. Physiol. 574 (Pt 2), 415-430

50 Destexhe, A. and Sejnowski, T.J. (2003) Interactions between membrane conductances underlying thalamocortical slow-wave oscillations. Physiol. Rev. 83, 1401-1453

51 Anderson, M.P. et al. (2005) Thalamic Cav3.1 T-type Ca2+ channel plays a crucial role in stabilizing sleep. Proc. Natl. Acad. Sci. U S A 102, 1743-1748
52 Tsakiridou, E. et al. (1995) Selective increase in T-type calcium conductance of reticular thalamic neurons in a rat model of absence epilepsy. J. Neurosci. 15, 3110-3117
53 Jeanmonod, D.et al. (1993) Thalamus and neurogenic pain: physiological, anatomical and clinical data. Neuroreport 4, 475-478
54 Kim, D. et al. (2003) Thalamic control of visceral nociception mediated by T-type Ca2+ channels. Science 302, 117-119
55 Hasler, W.L. (2004) Impaired thalamic T-type calcium channel function: a possible mechanism for visceral hyperalgesia and functional abdominal pain. Gastroenterology 126, 1912-1913
56 Willis, W.D. et al. (1999) A visceral pain pathway in the dorsal column of the spinal cord. Proc. Natl. Acad. Sci. U S A 96, 7675-7679
57 Snutch, T.P. (2005) Targeting chronic and neuropathic pain: the N-type calcium channel comes of age. NeuroRx 2, 662-670
58 Welsby, P.J. et al. (2003) A mechanism for the direct regulation of T-type calcium channels by Ca2+/calmodulin-dependent kinase II. J. Neurosci. 23, 10116-10121

# APPENDIX 2: ACTIVATION OF CORTICOTROPIN-RELEASING FACTOR RECEPTOR 1 SELECTIVELY INHIBITS CAv 3.2 T-TYPE CALCIUM CHANNELS* 

# Activation of Corticotropin-Releasing Factor Receptor 1 Selectively Inhibits Cav3.2 T-Type Calcium Channels ${ }^{\text {® }}$ 

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## ABSTRACT

The corticotropin-releasing factor (CRF) peptides CRF and urocortins 1 to 3 are crucial regulators of mammalian stress and inflammatory responses, and they are also implicated in disorders such as anxiety, depression, and drug addiction. There is considerable interest in the physiological mechanisms by which CRF receptors mediate their widespread effects, and here we report that the native CRF receptor 1 (CRFR1) endogenous to the human embryonic kidney 293 cells can functionally couple to mammalian Cav3.2 T-type calcium channels. Activation of CRFR1 by either CRF or urocortin (UCN) 1 reversibly inhibits Cav 3.2 currents ( $\mathrm{IC}_{50}$ of $\sim 30 \mathrm{nM}$ ), but it does not affect $\mathrm{Ca}_{\mathrm{v}} 3.1$ or Cav3.3 channels. Blockade of CRFR1 by the antagonist astressin abolished the inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.2$ channels. The

CRFR1-dependent inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.2$ channels was independent of the activities of phospholipase C , tyrosine kinases, $\mathrm{Ca}^{2+}$ /calmodulin-dependent protein kinase II, protein kinase C, and other kinase pathways, but it was dependent upon a cholera toxin-sensitive $G$ protein-mediated mechanism relying upon G protein $\beta \gamma$ subunits $(\mathrm{G} \beta \gamma)$. The inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.2$ channels via the activation of CRFR1 was due to a hyperpolarized shift in their steady-state inactivation, and it was reversible upon washout of the agonists. Given that UCN affect multiple aspects of cardiac and neuronal physiology and that $\mathrm{Ca}_{\mathrm{v}} 3.2$ channels are widespread throughout the cardiovascular and nervous systems, the results point to a novel and functionally relevant CRFR1-Cav3.2 T-type calcium channel signaling pathway.

The corticotropin-releasing factor (CRF) family, consisting of CRF, urocortin 1 (UCN), UCN2, and UCN3, are critical regulators of stress and inflammatory responses, and they have been variously associated with being cardioprotective and contributing toward alcohol and drug dependencies (Reul and Holsboer, 2002; Bale and Vale, 2004; Bruijnzeel and Gold, 2005; Gravanis and Margioris, 2005). The two

[^4]major receptors for CRF and UCNs, CRF receptor (CRFR) 1 and CRFR2, have been identified as G protein-coupled receptors (GPCRs) that can mediate responses via activation of the protein kinase signaling pathways (Bruijnzeel and Gold, 2005; Gravanis and Margioris, 2005). CRF has a higher affinity for CRFR1 than for CRFR2, UCN shows high affinity for both CRFR1 and CRFR2, whereas UCN2 and UCN3 are selective for CRFR2 (Bale and Vale, 2004). The CRFR1 is expressed primarily in the brain and pituitary, and activation of CRFR1 exerts numerous central and peripheral effects associated with pathological diseases (Dautzenberg and Hauger, 2002). Within the hypothalamus-pituitary axis, CRF and CRF-related peptides such as UCN activate CRFR1 receptors to regulate pituitary function in response to stress

ABBREVIATIONS: CRF, corticotropin-releasing factor; UCN, urocortin(s); GRFR, corticotropin-releasing factor receptor; GPCR, G proteincoupled receptor; HEK, human embryonic kidney; RGS, regulator of G protein signaling; MAS-GRK3, G protein-coupled receptor kinase; RT-PCR, reverse transcription-polymerase chain reaction; TBST, Tris-buffered saline/Tween 20; I-V, current-voltage; PTX, pertussis toxin; CTX, cholera toxin; GF-109203X, 3-[1-[3-(dimethylaminopropyl]-1H-indol-3-yl]-4-(1H-indol-3-yl)-1H-pyrrole-2,5-dione monohydrochloride; H-89, $N$-[2-(4-bromocinnamylamino)ethyl]-5-isoquinoline; KN-93, 2-(N-[2-hydroxyethyl])- $N$-(4-methoxybenzenesulfonyl)amino- N -(4-chlorocinnamyl)- N -methylamine; U-73122, 1-[6-[[17 $\beta$-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione; PKA, cAMP-dependent protein kinase; G $\beta \gamma, \mathrm{G}$ protein $\beta \gamma$ subunit; GDP $\beta$ S, guanosine-5'-O-(2-thiodiphosphate); GTP $\gamma$ S, guanosine-5'-O-(3-thiotriphosphate); PLC, phospholipase C; PKC, protein kinase C; TK, tyrosine kinase(s); ET-18- $\mathrm{OCH}_{3}$, 1-O-octadecyl-2-O-methyl-rac-glycero-3-phosphocholine; $\mathrm{CaMKII}^{2} \mathrm{Ca}^{2+} / \mathrm{calmodulin}-$ dependent protein kinase II; PI3K, phosphatidylinositol 3-kinase; RT, reverse transcriptase; PKI 5-24, protein kinase A inhibitor fragment 5-24.

[^5](Reul and Holsboer, 2002; Bale and Vale, 2004). In addition, overactive CRFR1 receptors in extrahypothalamic circuits have been implicated in affective disorders (Reul and Holsboer, 2002).
Low-voltage-activated calcium channels play critical roles in thalamocortical processes in both the awake and sleep states, pacemaking activity, action potential burst firing (Steriade and Llinas, 1988), and pain transmission and hormone secretion (Perez-Reyes, 2003; Hildebrand and Snutch, 2006). In mammals, three $\alpha_{1}$-subunit genes have been described that encode distinct low voltage-activated calcium channels (T-type) with unique biophysical and pharmacological properties: $\mathrm{Ca}_{\mathrm{V}} 3.1\left(\alpha_{1 \mathrm{G}}\right), \mathrm{Ca}_{\mathrm{V}} 3.2\left(\alpha_{1 \mathrm{H}}\right)$, and $\mathrm{Ca}_{\mathrm{V}} 3.3\left(\alpha_{1 \mathrm{I}}\right)$ (Perez-Reyes, 2003). Previous reports have shown that $\mathrm{Ca}_{\mathrm{v}} 3.1, \mathrm{Ca}_{\mathrm{V}} 3.2$, and $\mathrm{Ca}_{\mathrm{V}} 3.3$ are differentially and widely expressed in brain and various peripheral tissues (Cribbs et al., 1998; McKay et al., 2006; Molineux et al., 2006). Altered T-type calcium channel activity has been implicated in cardiac hypertrophy (Nuss and Houser, 1993), generalized epilepsies (Nelson et al., 2006), and both acute and chronic pain signaling (Bourinet et al., 2005). Although the electrophysiological properties of $\mathrm{Ca}_{\mathrm{V}} 3 \mathrm{~T}$-type channels are primarily regulated by dynamic changes in membrane potential, their functional properties can also be modulated by the actions of hormones or neurotransmitters acting via GPCRs that trigger downstream transduction pathways (Welsby et al., 2003; Wolfe et al., 2003; Chemin et al., 2006; Kim et al., 2006).
UCN, an endogenous agonist for CRF receptors, has been shown to either attenuate or stimulate low threshold calcium currents depending on the type of native cells examined (Lee and Tse, 1997; Tao et al., 2005; Kim et al., 2007). Various T-type calcium channel subtypes are expressed in these cells (Jagannathan et al., 2002); however, because of the lack of discriminatory antagonists, the selective inhibitory effect of CRFR1 activation on each of the three $\mathrm{Ca}_{\mathrm{v}} 3$ channels- $\mathrm{Ca}_{\mathrm{v}} 3.1, \mathrm{Ca}_{\mathrm{v}} 3.2$, and $\mathrm{Ca}_{\mathrm{v}} 3.3$-could not be investigated pharmacologically in these cells. As CRFR1, $\mathrm{Ca}_{\mathrm{V}} 3$ and various calcium channels are localized to peripheral and central neuron regions, including the amygdala, hippocampus, hypothalamus, and pituitary (Chalmers et al., 1995; Talley et al., 1999), it is of interest to ask by what mechanism does the activation of the CRFR1 underline the inhibition of $\mathrm{Ca}_{\mathrm{v}} 3$ channels and to ask whether the $\mathrm{Ca}_{\mathrm{v}} 3$ calcium channels are specifically inhibited.
In the present study, we report that activation of CRFR1 endogenous to the HEK293 cells selectively inhibited $\mathrm{Ca}_{\mathrm{V}} 3.2$ calcium channels, whereas $\mathrm{Ca}_{\mathrm{v}} 3.1$ and $\mathrm{Ca}_{\mathrm{v}} 3.3$ channels were not affected. Besides, the mechanism for the inhibition was mediated via a cholera-toxin sensitive, $\mathrm{G} \beta \gamma$-dependent pathway. It is noteworthy that the decrease in $\mathrm{Ca}_{\mathrm{v}} 3.2$ currents was due to a novel mechanism involving the hyperpolarized shift in the steady-state inactivation property of the channels, and the effect is reversible upon washout of the CRFR1 agonists. As UCN affect multiple aspects of cardiac and neuronal physiology and as $\mathrm{Ca}_{\mathrm{v}} 3.2$ channels are expressed widely throughout the cardiovascular and nervous systems, our results point to a novel and functionally relevant CRFR1calcium channel signaling pathway.

## Materials and Methods

Cell Culture and Transient Transfection Protocols. HEK293 cells were maintained in Dulbecco's modified Eagle's medium $+10 \%$
fetal bovine serum. Transient transfection was performed using the standard calcium phosphate transfection method with a DNA mix containing 1:9 ratios (by weight) of green fluorescent protein plasmid and constructs encoding for rat or human $\mathrm{Ca}_{\mathrm{v}} 3.1, \mathrm{Ca}_{\mathrm{v}} 3.2$, and $\mathrm{Ca}_{\mathrm{v}} 3.3$ isoforms. The full-length rat (McRory et al., 2001) and human (kindly provided by David Parker, Neuromed Pharmaceuticals, Inc., Vancouver, BC , Canada) $\mathrm{Ca}_{\mathrm{v}} 3.1, \mathrm{Ca}_{\mathrm{v}} 3.2$, and $\mathrm{Ca}_{\mathrm{v}} 3.3 \alpha_{1}$-subunits were cloned into the pcDNA3 vector (Invitrogen, Carlsbad, CA). RGS2 and MASGRK3 constructs (both kindly provided by Dr. Brett Adams, Utah State University, Logan, UT) were subcloned into pEGFP-C2 and pIRES vectors, respectively (Clontech, Mountain View, CA).

Reverse Transcription-Polymerase Chain Reaction. Total RNA was extracted from HEK293 cells using the RNeasy kit (QIAGEN, Valencia, CA). Reverse transcription was carried out with SuperScript II (Invitrogen). Negative control (reactions without reverse transcriptase) was carried out in all RT-PCRs to exclude contamination. The sequences of the primers used in this study are summarized in Supplemental Table 1. Hypothalamus and left cardiac atrium were used as positive controls for the expression of CRFR2 $\alpha$ and CRFR2 $\beta$, respectively. The expression of $\beta$-actin mRNA was examined as an internal control. The PCR protocol includes a denaturation step at $95^{\circ} \mathrm{C}$ for 2 min , and the denaturation, annealing, and elongation cycle was carried out at $94^{\circ} \mathrm{C}$ for 30 s , at $65^{\circ} \mathrm{C}$ (CRFR1, R2 $\alpha$, and R2 $\beta$ ) or $60^{\circ} \mathrm{C}$ ( $\beta$-actin) for 20 s , and at $72^{\circ} \mathrm{C}$ for 1 min. PCR was carried out for 35 cycles (CRFR1, R2 $\alpha$, and R2 $\beta$ ) or 25 cycles ( $\beta$-actin), respectively. PCR analysis was repeated at least twice with the same samples to confirm reproducibility of the results.

Western Blotting. HEK293 cells were lysed for 1 h in buffer containing $1 \%$ Triton $\mathrm{X}-100,10 \mu \mathrm{~g} / \mathrm{ml}$ aprotinin, and 1 mM phenylmethylsulfonyl fluoride in phosphate-buffered saline (PBS). After centrifugation at $40,000 \mathrm{rpm}$ for 30 min at $4^{\circ} \mathrm{C}, 30 \mu \mathrm{~g}$ of soluble protein was separated in $10 \%$ SDS-polyacrylamide gel. The proteins were then transferred electrophoretically onto polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA) using a semidry transfer system (Bio-Rad Laboratories) with methanol omitted from the transfer buffer. For immunolabeling experiments, membranes were first incubated with $5 \%$ nonfat milk in TBST ( 20 mM Tris, $\mathrm{pH} 7.6,137 \mathrm{mM} \mathrm{NaCl}$, and $0.05 \%$ Tween 20) for 1 h at room temperature. The membranes were then incubated with diluted primary antibody anti-CRFR1 (1:500 dilution, V-14; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or anti-CRFR2 (1:500 dilution, C-15; Santa Cruz Biotechnology, Inc.) at $4^{\circ} \mathrm{C}$ overnight. After five washes with TBST, membranes were incubated for 2 h with 2000fold diluted rabbit anti-goat secondary antibody (Sigma-Aldrich, St. Louis, MO). After another five washes with TBST, the specific binding of the primary antibody was detected with SuperSignal West Pico chemiluminescent substrate (Pierce Chemical, Rockford, IL). The intensities of immunoreactive staining were measured using a scanning densitometer (Scion Image; Scion Corporation, Frederick, MD).

Immunohistochemical Localization of CRFR1. HEK293 cells were grown on polylysine-coated sterile coverslips, and then they were fixed in PBS containing $4 \%$ sucrose and $4 \%$ paraformaldehyde for 20 min at $4^{\circ} \mathrm{C}$. The fixed cells were washed three times with PBS before permeabilization in PBS containing $0.1 \%$ Triton X-100 for 5 $\min$. Blockade was then carried out with $4 \%$ horse serum in PBS for 1 h . This was followed by incubation in primary antibody (goat anti-CRFR1, 1:500; Santa Cruz Biotechnology, Inc.) for 1 h at $25^{\circ} \mathrm{C}$. After washing three times with PBS, Alexa Fluor 488 chicken antigoat IgG (green) (Invitrogen) was applied to the samples at a dilution of $1: 500$. The immunolabeled cells were visualized using a laserscanning confocal microscope (Fluoview BX61; Olympus, Tokyo, Japan). Negative controls, omitting each primary antibody, were performed in each case, and no significant immunolabelings were observed (data not shown).
Measurement of cAMP. To determine intracellular cAMP levels, 0.5 mM 3-isobutyl-1-methylxanthine, a cyclic nucleotide phosphodiesterase inhibitor, was added to each well 30 min before the addition of CRF, UCN, or astressin to prevent breakdown of accumulated cAMP.

## Appendix 2: Activation of CRFR1 selectively inhibits $\mathrm{Ca}_{\mathrm{V}} 3.2$

After incubation with CRF or UCN for 10 min , cells were immediately immersed in 0.25 ml of 0.1 M HCl to stop the reaction. For experiments using antagonist, the astressin was applied for 30 min before the 10 min incubation as described above. Cells were collected and then centrifuged at 3000 rpm for 15 min at room temperature. The intracellular cAMP content was determined from the supernatant using the direct cAMP enzyme immunoassay kit in accordance with the manufacturer's high-sensitivity acetylation protocol (Sigma-Aldrich).
Cloning of Rat CRFR1 Receptor. Rat CRFR1 mRNA was isolated from rat (Wistar) brain, and RT-PCR was performed using CRFR1 forward (5-ATGGGACGGCGCCCGCAGCTCCGGCTCG-3) and reverse (5-TCACACTGCTGTGGACTGCTTGATGC-3) primers. The PCR product was cloned into pGEM-T Easy (Promega, Madison, WI) vector, and the identity of the rat CRFR1 was confirmed by DNA sequencing. The full-length rat CRFR1 digested with restriction enzymes SpeI and NotI was subcloned into MCS B site of the pIRES vector (Clontech), whereas mCherry (gift from Dr. Roger Y. Tsien, University of California, San Diego, CA) was cloned into the MCS A site using the XhoI and EcoRI restriction enzyme sites.
Electrophysiological Recordings and Data Analysis. Wholecell currents were recorded at room temperature. Extracellular solution contained 140 mM tetraethylammonium methanesulfonate, 10 mM HEPES, and $5 \mathrm{mM} \mathrm{BaCl} 2(\mathrm{pH}$ to 7.3 with CsOH$)$. Patch pipettes (WPI, Sarasota, FL) have a resistance of 2 to $3 \mathrm{M} \Omega$ when filled with an internal solution of $138 \mathrm{mM} \mathrm{Cs}-\mathrm{MeSO}_{3}, 5 \mathrm{mM} \mathrm{CsCl}, 0.5$ EGTA, $1 \mathrm{mM} \mathrm{MgCl} 2,4 \mathrm{mM} \mathrm{MgATP}$, and 10 mM HEPES ( pH 7.3 , adjusted with CsOH ). Complete replacement of external solution 2 $\mathrm{ml} / \mathrm{min}$ in the chamber was achieved within 2 to 3 min . Whole-cell $\mathrm{Ba}^{2+}$ currents were recorded using a MultiClamp 700B amplifier (Axon Instruments, Foster City, CA), controlled and monitored with a PC running p-CLAMP software, version 9.3 (Axon Instruments). Series resistance was typically $<5 \mathrm{M} \Omega$, and then it was electronically compensated by at least $70 \%$. Data were low pass-filtered at 2 kHz using the built-in Bessel filter of the amplifier, and subtraction of capacitance and leakage current was carried out on-line using the $\mathrm{P} / 4$ protocol. Student's $t$ tests were used to compare the different values, and they were considered significant at $P<0.05$. All data are expressed as means $\pm$ S.E.M., and GraphPad Prism software (GraphPad Software Inc., San Diego, CA) was used for data plotting. Concentration-response curves were fitted by sigmoidal Hill equation $I / I_{\text {control }}=1 /\left(1+10^{\left(\log I C_{50-X)}\right.} n_{\mathrm{H}}\right)$, where $X$ is the decadic logarithm of the concentration used, $\mathrm{IC}_{50}$ is the concentration at which the half-maximum effect occurs, and $n_{\mathrm{H}}$ is the Hill coefficient. The current-voltage (I-V) curves were fitted by $\mathrm{I}_{\mathrm{Ba}}=G_{\text {max }}\left(V-E_{\text {rev }}\right) /\{1+$ $\left.\exp \left(\left(V-V_{1 / 2}\right) / k_{\text {I-v }}\right)\right\}$. Activation data were fitted by $G / G_{\max }=F_{\text {low }} /$ $\left\{1+\exp \left(\left(V_{1 / 2, \text { low }}-V\right) / k_{\text {low }}\right)\right\}+\left(1-F_{\text {low }}\right) /\left\{1+\exp \left(\left(V_{1 / 2, \text { high }}-V\right) / k_{\text {high }}\right)\right\}$, where $V_{1 / 2 \text { act }}$ is the potential for half-activation calculated from dual Boltzmann functions when $G=0.5 G_{\text {max }}$. Steady-state inactivation data were fitted by a Boltzmann function of the form $I / I_{\max }=(\mathrm{A} 1-$ $\mathrm{A} 2) /\left\{1+\exp \left(\left(V-V_{1 / 2 \text { inact }}\right) / k_{\text {inact }}\right)\right\}+\mathrm{A} 2$. Recovery of currents from inactivation was tested using a double-pulse protocol. The peak current from the second test pulse was always normalized to the first 1-s pulse, and the plot of normalized current versus repolarization time was fitted with a double exponential equation: $Y=Y_{\text {min }}+\mathrm{A}_{1} \times$ $\left[1-\exp \left(-t / \tau_{\mathrm{f}}\right)\right]+\mathrm{A}_{2} \times\left[1-\exp \left(-t / \tau_{\mathrm{s}}\right)\right]$, where $Y$ is the fraction of recovery, $\mathrm{A}_{1}$ and $\mathrm{A}_{2}$ are the maximum values of the fast and slow component, and $\tau_{\mathrm{f}}$ and $\tau_{\mathrm{s}}$ are the time constants, respectively.
Pharmacological Agents. All drugs were obtained from SigmaAldrich, unless otherwise indicated. Stock solutions of UCN, CRF, PKI 5-24, chelerythrine chloride, pertussis toxin (PTX), cholera toxin (CTX), and astressin were prepared in distilled deionized water. Stock solutions of GF109203X, H-89, wortmannin, genistein, KN-93, and U-73122 were prepared in dimethyl sulfoxide. The concentration of dimethyl sulfoxide in the bath solution is expected to be less than $0.01 \%$, and it had no functional effects on the T-type calcium currents. The QEHA peptide (QEHAQEPERQYMHIGTMVEFAYALVGK) and SKEE peptide (SKEEKSDKERWQHL ADLADFALAMKDT) were synthesized by GenScript Corporation (Scotch Plains, NJ). The pep-
tides were purified by high-performance liquid chromatography ( $>95 \%$ ), and the identities were verified by mass spectrometry.

## Results

HEK293 Cells Endogenously Expressed Functional
CRFR1. Previous reports have shown that HEK293 cells endogenously express CRF receptors (Dautzenberg et al., 2000). To confirm both the receptor subtype and the ability of endogenous receptors to transduce downstream signals after binding CRF or UCN, we characterized HEK293 cells by RT-PCR, Western blot, and immunohistochemistry analyses.
RT-PCR analysis demonstrated that CRFR1 mRNA was expressed in HEK293 cells, but that neither CRFR2 $\alpha$ nor CRFR2 $\beta$ mRNA could be detected (Fig. 1A). As controls for the RT-PCR, CRFR2 $\alpha$ and CRFR2 $\beta$ transcripts could be clearly amplified from mRNAs isolated from mouse hypothalamus and left atrium, respectively (Fig. 1A). All RT-PCR reactions were able to amplify $\beta$-actin, whereas the negative controls performed in parallel in the absence of reverse transcriptase enzyme in the RT reaction showed no product. Western blot analysis of HEK293 cell protein lysates using anti-CRFR1 antibody revealed that CRFR1 was expressed at the predicted size of $\sim 55 \mathrm{kDa}$, whereas CRFR2 was not detected by anti-CRFR2 antibody (Fig. 1B). As a positive control, mouse hypothalamus, which is known to express both CRFR1 and CRFR2, showed prominent bands of $\sim 55$ kDa (CRFR1) and $\sim 65 \mathrm{kDa}$ (CRFR2), and both HEK293 cell and hypothalamus lysates produced robust staining for $\beta$-actin (Fig. 1B). As CRF receptors belong to the family of seventransmembrane GPCRs (Markovic et al., 2006) and would be expected to be localized to the plasma membrane, we examined the distribution of CRFR1 on HEK293 cells by confocal microscopy. Figure 1C clearly shows the membrane localization of CRFR1. To test the functionality of the endogenous CFRR1 in HEK293 cells, we measured the levels of cAMP produced from CRFR1 activation by either CRF or UCN, both of which are known to activate adenylate cyclase (Markovic et al., 2006). Upon either $0.01 \mu \mathrm{M}$ CRF or $0.01 \mu \mathrm{M} \mathrm{UCN}$ application to HEK293 cells, cAMP production was stimulated $\sim 7$-fold over basal levels (Fig. 1D). In the presence of the CRFR1 antagonist, $1 \mu \mathrm{M}$ astressin, the stimulation of cAMP accumulation by UCN or CRF was completely abrogated (Fig. 1D). Taken together, the results show that the HEK293 cells endogenously expressed fully functional CRFR1 and that they could therefore be used to investigate the mechanisms by which $\mathrm{Ca}_{\mathrm{V}} 3$ T-type calcium channels might be affected.

CRFR1 Selectively Inhibited Cave 3.2 T-Type Calcium Channels. The binding affinities of CRF and UCN peptides to CRFR1 are roughly similar (Dautzenberg et al., 2000), and we decided to first investigate the activation of CRFR1 by UCN on $\mathrm{Ca}_{\mathrm{v}} 3$ T-type calcium channels. Figure 1 shows that rat $\mathrm{Ca}_{\mathrm{V}} 3.2$ channels were selectively inhibited by application of $0.1 \mu \mathrm{M} \mathrm{UCN}$, whereas the rat $\mathrm{Ca}_{\mathrm{V}} 3.1$ and $\mathrm{Ca}_{\mathrm{V}} 3.3$ T-type calcium channels were not affected. A similar selective inhibition of $\mathrm{Ca}_{\mathrm{V}} 3.2$ channels was observed at all test potentials (Fig. 2, A-C, right). Overall, application of $0.1 \mu \mathrm{M} \mathrm{UCN}$ significantly reduced $\mathrm{Ca}_{\mathrm{V}} 3.2$ currents by $\sim 31 \%$ ( $I / I_{\text {control }}=$ $0.70 \pm 0.05, n=15, P<0.05)$, whereas neither $\mathrm{Ca}_{\mathrm{V}} 3.1$ nor $\mathrm{Ca}_{\mathbf{V}} 3.3$ currents were significantly affected $\left(\mathrm{Ca}_{\mathrm{V}} 3.1\right.$ : $I / I_{\text {control }}=0.98 \pm 0.03, n=13, P>0.05 ; \mathrm{Ca}_{\mathrm{V}} 3.3: I / I_{\text {control }}=$
$0.96 \pm 0.04, n=6, P>0.05)$. After washout, $\mathrm{Ca}_{\mathrm{V}} 3.2$ channel currents returned to baseline levels within 5 min (Fig. 2F), indicating the effect of UCN on $\mathrm{Ca}_{\mathrm{V}} 3.2$ currents was not due to rundown. To determine whether the observed selective inhibition of $\mathrm{Ca}_{\mathbf{v}} 3$ was species-specific, we further investigated the effects of $0.1 \mu \mathrm{M} \mathrm{UCN}$ on the three human brain $\mathrm{Ca}_{\mathrm{V}} 3$ T-type calcium channel isoforms expressed in HEK293 cells. Figure 1 E shows a similar degree of UCN-mediated inhibition $(\sim 37 \%)$ of human $\mathrm{Ca}_{\mathrm{V}} 3.2$ currents $\left(I / I_{\text {control }}=\right.$ $0.63 \pm 0.06, n=7, P<0.01$ ), with no significant effects on either $\mathrm{Ca}_{\mathrm{V}} 3.1\left(I / I_{\text {control }}=0.97 \pm 0.03, n=9, P>0.05\right)$ or $\mathrm{Ca}_{\mathrm{V}} 3.3\left(I / I_{\text {control }}=0.98 \pm 0.07, n=7, P>0.05\right)$ currents. Electrophysiological recordings for the UCN-mediated inhibition of human $\mathrm{Ca}_{\mathrm{v}} 3$ T-type calcium channels and the time course of the inhibition are shown (Supplemental Fig. 8).

CRFR1 Activation Affected Cavi 3.2 Channels by Shifting Steady-State Inactivation Properties. Before investigating the mechanism for the selective inhibition of
$C a_{V} 3.2$ calcium channels via UCN activation of CRFR1, we examined 1) whether the response is dosage-dependent and 2) whether channel electrophysiological properties were affected. To address the first issue, we performed a UCN doseresponse study for the inhibition of $\mathrm{Ca}_{\mathbf{v}} 3.2$ currents. At a holding potential of -110 mV , application of UCN at 0.01 , $0.1,1$, and $10 \mu \mathrm{M}$ increasingly inhibited $\mathrm{Ca}_{\mathrm{V}} 3.2$ currents by $15 \%\left(I / I_{\text {control }}=0.85 \pm 0.03, n=8, P<0.05\right), 31 \%$ $\left(I / I_{\text {control }}=0.70 \pm 0.05, n=15, P<0.05\right), 41 \%\left(I / I_{\text {control }}=\right.$ $0.59 \pm 0.04, n=9, P<0.01)$, and $43 \%\left(I / I_{\text {control }}=0.57 \pm\right.$ $0.05, n=7, P<0.01$ ), respectively (Fig. 3A). The maximum inhibition reduced the original peak current by $\sim 43 \%$, with a calculated $\mathrm{IC}_{50}=30.41 \mathrm{nM}$ and a Hill coefficient $=0.98$. We next evaluated the inhibition of UCN at a more physiological holding potential of -80 mV , and we found that the $\mathrm{Ca}_{\mathrm{V}} 3.2$ channels were even more sensitive to inhibition by UCN with an $\sim 4$-fold decrease in the $\mathrm{IC}_{50}$ to 6.93 nM (Hill coefficient of 0.97; Fig. 3B). To address the second point, we investigated


Fig. 1. CRFR1 is endogenously expressed in HEK293 cells. A, detection of CRFR1, CRFR2 $\alpha$, and CRFR2 $\beta$ in HEK293 cells. Total RNA from mouse hypothalamus or left atrium was used as positive controls for CRFR2 $\alpha$ and CRFR2 $\beta$, respectively. Negative control indicates RT-PCR reaction without the addition of RT. B, Western blot analysis of CRFR1 (top), CRFR2 (middle), and $\beta$-actin (bottom) of HEK293 membrane protein extracts. Mouse hypothalamus expressing both CRFR1 ( $\sim 55 \mathrm{kDa}$ ) and CRFR2 ( $\sim 65 \mathrm{kDa}$ ) is used as positive control, whereas $\beta$-actin expression controls for loading. Quantifications of band intensities are measures of relative expression levels. C, membrane expression of CRFR1 determined by confocal microscopy. Merged picture (top) of bright field (bottom, left) and fluorescent signals of CRFR1 (bottom, right). Scale bar, $20 \mu \mathrm{~m}$. Proteins were immunolabeled by a monoclonal goat anti-CRFR1 antibody (green; Alexa). D, application of $0.01 \mu \mathrm{M} \mathrm{UCN}$ and $0.01 \mu \mathrm{M} \mathrm{CRF}$ stimulated robust cAMP production, but not in the presence of antagonist astressin at $1 \mu \mathrm{M}$. The results are representative of three independent experiments. *, $P<0.05$ versus control.

Appendix 2: Activation of CRFR1 selectively inhibits Ca 3.2
whether the steady-state activation and inactivation properties of $\mathrm{Ca}_{\mathrm{v}} 3.2$ channels were affected by bath application of $0.1 \mu \mathrm{M}$ UCN. We observed a slight but statistically insignificant shift of 2 mV in the hyperpolarizing direction of the activation potential ( $V_{1 / 2}$ from $-41.8 \pm 2.6$ to $-43.7 \pm 1.3 \mathrm{mV}$ and $k$ value from $5.9 \pm 0.3$ to $6.0 \pm 0.6, n=19, P>0.05$ ) (Fig. 3C). In contrast, $0.1 \mu \mathrm{M}$ UCN significantly shifted the
steady-state inactivation potentials of $\mathrm{Ca}_{\mathrm{v}} 3.2$ calcium channels in the hyperpolarizing direction by $\sim 13 \mathrm{mV}$ ( $V_{1 / 2}$ from $-66.7 \pm 0.6$ to $-79.5 \pm 0.7 \mathrm{mV}$, and $k$ value from $4.9 \pm 0.6$ to $7.2 \pm 0.7, n=17, P<0.05$ ) (Fig. 3D). In addition, UCN at $30 \mathrm{nM}\left(\sim \mathrm{IC}_{50}\right.$ at holding potential of $\left.-110 \mathrm{mV}\right)$ shifted the steady-state inactivation potentials of $\mathrm{Ca}_{\mathrm{v}} 3.2$ calcium channels in the hyperpolarizing direction by an expected smaller

A


B



D




Fig. 2. Effects of UCN-mediated CRFR1 activation on cloned T-type $\mathrm{Ca}_{\mathrm{v}} 3$ calcium channels. A to C, left, representative traces showing the effect of $0.1 \mu \mathrm{M} \mathrm{UCN}$ (black triangles) on $5 \mathrm{mM} \mathrm{Ba}{ }^{2+}$ currents elicited by a $-30-\mathrm{mV}$ test pulse. Right, I-V profiles (evoked by a series of depolarizing pulses from a holding potential of -110 mV to test potentials between -90 and 0 mV , in $10-\mathrm{mV}$ increments) obtained for cloned rat $\mathrm{Ca}_{\mathrm{v}} 3.1$ ( $\mathrm{A} ; n=13$ ), $\mathrm{Ca}_{\mathrm{v}} 3.2$ ( $\mathrm{B} ; n=15$ ), and $\mathrm{Ca}_{\mathrm{v}} 3.3$ subunits (C; $n=6$ ). D, effect of $0.1 \mu \mathrm{M} \mathrm{UCN}$ on $\mathrm{I}_{\mathrm{Ba}}$ flowing through cloned rat $\mathrm{Ca}_{\mathrm{v}} 3.1, \mathrm{Ca}_{\mathrm{v}} 3.2$, and $\mathrm{Ca}_{\mathrm{v}} 3.3$ channels elicited by a $-30-\mathrm{mV}$ test pulse. E, effect of $0.1 \mu \mathrm{M} \mathrm{UCN}$ on $\mathrm{I}_{\mathrm{Ba}}$ flowing through cloned human $\mathrm{Ca}_{\mathrm{V}} 3.1(n=9)$, $\mathrm{Ca}_{\mathrm{v}} 3.2(n=7)$, and $\mathrm{Ca}_{\mathrm{v}} 3.3(n=7)$ channels elicited by a $-30-\mathrm{mV}$ test pulse. F, exemplary traces (left) and time course (right) of $\mathrm{Ca}_{\mathrm{v}} 3.2$ currents obtained before (control), during, and after (washout) the application of $0.1 \mu \mathrm{M}$ UCN. After washout, $\mathrm{Ca}_{\mathrm{v}} 3.2$ currents recorded in control cells returned to original amplitude.

## Appendix 2: Activation of CRFR1 selectively inhibits Ca 3.2

potential of $\sim 8 \mathrm{mV}\left(V_{1 / 2}=-75.3 \pm 0.7 \mathrm{mV}\right.$ and $k=6.6 \pm 0.8$, $n=6, P<0.05$ versus control) (Fig. 3E), suggesting a dose dependence in the shift of the steady-state inactivation upon activation of CRFR1 by UCN. Furthermore, after $0.1 \mu \mathrm{M}$ UCN washout, the steady-state inactivation reversed to values similar to controls before UCN application $\left(V_{1 / 2}=\right.$ $-68.4 \pm 0.7$ and $k=5.2 \pm 0.6, n=8, P<0.05)$. This result suggests that the reduction in $\mathrm{Ca}_{\mathrm{v}} 3.2$ currents observed upon
application of UCN could be the result of increased channels remaining in the inactivated state. We further determined whether activation of CRFR1 will affect $\mathrm{Ca}_{\mathrm{V}} 3.2$ recovery from inactivation. A typical two-pulse protocol was used with a prepulse of 1-s duration (Fig. 3F). We observed a slight but statistically insignificant effect of $0.1 \mu \mathrm{M} \mathrm{UCN}(P>0.05)$ on the fast and slow components of recovery from inactivation (control: $\tau_{\text {fast }}=110 \pm 19 \mathrm{~ms}, n=9, \tau_{\text {slow }}=916 \pm 113 \mathrm{~ms}, n=$

A


E
F


Fig. 3. Dose response and effects of UCN on $\mathrm{Ca}_{\mathrm{v}} 3.2$ channel biophysical properties. A and $B$, concentration-dependent effects of UCN on $\mathrm{Ca}_{\mathrm{v}} 3.2$ currents. $\mathrm{Ca}_{\mathrm{v}} 3.2$ currents were evoked by a $100-\mathrm{ms}$ depolarizing step to -30 mV from a holding potential of $-110 \mathrm{mV}(\mathrm{A})$ and -80 mV (B). Dose-response curves were established by fitting the normalized currents with a sigmoidal Hill equation, where $I / I_{\text {control }}=1 /\left(1+10^{(\log \text { IC50-|supi|X) }} n_{\mathrm{H}}\right)$ (left; see Supplemental Material). Exemplary traces in the absence and presence of UCN on evoked $\mathrm{Ca}_{\mathrm{v}} 3.2$ currents are shown (right). C, effects of $0.1 \mu \mathrm{M}$ UCN on the steady-state activation curve of $\mathrm{Ca}_{\mathrm{v}} 3.2$. Exemplary tail currents evoked by repolarizations to -100 mV after depolarizing test pulses at -90 , -60 , 0 , or 20 mV (top) and normalized steady-state activation curves (bottom) in the absence (■) and presence ( $\mathbf{\Delta}$ ) of UCN. Steady-state activation curves were activated by a variant voltage family of $20-\mathrm{ms}$ test pulses, from -90 to 20 mV , and tail currents were recorded on repolarization to -100 mV . D, effect of $0.1 \mu \mathrm{M}$ UCN on the steady-state inactivation curve of $\mathrm{Ca}_{\mathrm{v}} 3.2$. Typical current traces after 15-s conditioning depolarizing pulses evoked at $-120,-90$, -60 , or -30 mV (top) and normalized steadystate inactivation curves (bottom) in the absence (■) and presence ( $\mathbf{\Lambda}$ ) of UCN, and washout (O). Steady-state inactivation curves were obtained by evoking $\mathrm{Ca}_{\mathrm{v}} 3.2$ currents with a test depolarization to -30 mV applied at the end of $15-\mathrm{s}$ conditioning pulses ranging from -120 to -30 mV . E, effect of 30 nM UCN on the steady-state inactivation of $\mathrm{Ca}_{\mathrm{v}} 3.2(n=6)$. F, effect of $0.1 \mu \mathrm{M} \mathrm{UCN}$ on the recovery from inactivation of $\mathrm{Ca}_{\mathrm{v}} 3.2$ channels. Double-pulse protocol started from a holding potential of -110 mV , with a 1 -s depolarizing prepulse to -30 mV , following by a variable length (from 10 ms to 5.12 s ) of repolarization to -110 mV , and finally a second $50-\mathrm{ms}$ depolarizing pulse to -30 mV . Sweeps were separated by 20 s to allow for complete channel recovery. Fraction recovery was calculated as the ratio of the peak current measured during the second test pulse divided by the peak current measured during the prepulse.
$9 ; 0.1 \mu \mathrm{M} \mathrm{UCN}: \tau_{\text {fast }}=103 \pm 17 \mathrm{~ms}, n=9, \tau_{\text {slow }}=684 \pm 72$ $\mathrm{ms}, n=9$ ) (Fig. 3F), which suggested that the UCN blockade purely shifted the steady state of inactivation potentials to the left and that it did not affect the rate of recovery. Interestingly, however, there seemed to be some facilitation of the $\mathrm{Ca}_{\mathrm{V}} 3.2$ channels under both conditions.
UCN and CRF Similarly Inhibited Ca 3 3.2 Currents by Activation of CRFR1. To confirm that the UCN-mediated response occurred via CRFR1, we examined its effects on $\mathrm{Ca}_{\mathrm{V}} 3.2$ currents in the presence or absence of the CRFR antagonist astressin. Preincubation with $1 \mu \mathrm{M}$ astressin blocked the UCN-induced inhibition $\left(I / I_{\text {control }}=0.95 \pm 0.06\right.$, $n=6, P<0.05$ ) (Fig. 4A), indicating that the inhibition of $\mathrm{Ca}_{\mathrm{V}} 3.2$ channels is CRFR1-dependent. Importantly, CRF peptide activation of CRFR1 also showed robust inhibition (by $\sim 28 \%$ ) of the $\mathrm{Ca}_{\mathrm{V}} 3.2$ currents $\left(I / I_{\text {control }}=0.72 \pm 0.04\right.$, $n=6, P<0.05$ ) (Fig. 4B) and was CRFR1-dependent as demonstrated by the abrogation of inhibition after preincubation with $1 \mu \mathrm{M}$ astressin.
$\mathrm{Ca}_{\mathrm{v}}$ 3.2 Channel Inhibition Can Be Enhanced by Exogenous CRFR1 Expression. We next explored whether augmentation of CRFR1 levels through the overexpression of cloned CRFR1 in HEK293 cells could affect the degree of inhibition of $\mathrm{Ca}_{\mathrm{V}} 3.2$ channels mediated by UCN or CRF. We first cloned and then characterized the rat CRFR1 in HEK293 cells. Western blot analysis of CRFR1 proteins isolated from mouse hypothalamus and CRFR1-transfected HEK293 lysates showed a predominant $\sim 55-\mathrm{kDa}$ band stained by anti-CRFR1 (Fig. 5A). The total amount of CRFR1 protein expressed in the HEK293 cells after transient transfection with cloned CRFR1 showed a 3 - to 4 -fold increase over the endogenous level, after normalization with the $\beta$-actin
expression level (Figs. 1B and 5A). Application of $0.01 \mu \mathrm{M}$ UCN or $0.01 \mu \mathrm{M}$ CRF on HEK293 cells transfected with the cloned CRFR1 stimulated cAMP production by approximately 80 -fold over basal levels ( $82.91 \pm 6.40$ for CRF and $79.29 \pm 6.37$ for UCN). This represented an $\sim 10$-fold increase in cAMP compared with that for nontransfected HEK293 cells (Figs. 1D and 5B). In the presence of the CRFR1 antagonist astressin at $1 \mu \mathrm{M}$, the level of cAMP production after application of either UCN or CRF was not significantly different from basal level exhibited by control HEK293 cells (Fig. 5B).

We also examined whether the level of $\mathrm{Ca}_{\mathrm{V}} 3.2$ channel inhibition might be increased in the CRFR1-transfected HEK293 cells. Figure 5C shows that upon application of either $0.1 \mu \mathrm{M}$ UCN or CRF that $\mathrm{Ca}_{\mathbf{V}} 3.2$ currents were significantly reduced by $46.3 \%\left(I / I_{\text {control }}=0.54 \pm 0.04, n=6\right.$, $P<0.05)$ and $42.5 \%\left(I / I_{\text {control }}=0.57 \pm 0.04, n=6, P<0.05\right)$, respectively. These values represent an $\sim 50 \%$ increase in the degree of inhibition of $\mathrm{Ca}_{\mathrm{V}} 3.2$ currents compared with that for non-CRFR1-transfected HEK293 cells. Dose-response analysis of UCN inhibition in CRFR1 transfected HEK293 cells showed an enhanced affinity by $\sim 6$-fold $\left(\mathrm{IC}_{50}=5.07 \mathrm{nM}\right.$ and Hill coefficient $=0.96)($ Fig. 5C, bottom). Again, pretreatment of the cells with $1 \mu \mathrm{M}$ astressin abrogated the inhibitory effects of UCN and CRF on Ca 3.2 currents $\left(I / I_{\text {control }}=\right.$ $0.95 \pm 0.03$ for UCN, $n=6$ and $I / I_{\text {control }}=0.96 \pm 0.04$ for CRF, $n=6$ ) (Fig. 5, C and D). Overall, the apparent nonlinearity of the relationship between the level of CRFR1 protein, the production of cAMP , and the degree of $\mathrm{Ca}_{\mathrm{V}} 3.2$ inhibition may reflect the fact that cAMP/cAMP-dependent protein kinase (PKA)-dependent intracellular signaling factors required to affect inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.2$ channels might be lim-


Fig. 4. UCN and CRF inhibit $\mathrm{Ca}_{\mathrm{v}} 3.2$ currents via activation of CRFR1. Ca 3.2 currents were evoked by a $100-\mathrm{ms}$ depolarizing step to -30 mV from a holding potential of -110 mV . Cells were incubated with $1 \mu \mathrm{M}$ astressin for 30 min before application of UCN or CRF. A, exemplary traces (left) and pooled data (right) show the effects of $0.1 \mu \mathrm{M}$ UCN on $\mathrm{Ca}_{\mathrm{y}} 3.2$ currents in the presence of 1 $\mu \mathrm{M}$ astressin. B, exemplary traces (top) and pooled data (bottom) show the effects of 0.1 $\mu \mathrm{M} \mathrm{CRF}$ on $\mathrm{Ca}_{\mathrm{v}} 3.2$ currents in the absence (left) and presence (right) of astressin.
iting. Alternatively, it may indicate that a signaling mechanism distinct from the well described CRFR1-mediated cAMP/PKA pathway is involved in the inhibition of $\mathrm{Ca}_{\mathrm{V}} 3.2$ T-type channels (see below).

Involvement of $G$ Proteins in CRFR1-Mediated $\mathbf{C a}_{\mathbf{v}}$ 3.2 Calcium Channel Inhibition. To address whether G proteins are directly involved in the CRFR1-dependent inhibition of $\mathrm{Ca}_{\mathrm{V}} 3.2$ calcium channels, we dialyzed into cells guanosine-5'-O-(2-thiodiphosphate) (GDP $\beta$ S; 1 mM ), a nonhydrolysable GDP analog. It shows that GDP $\beta$ S abolished
the inhibition of $\mathrm{Ca}_{\mathrm{V}} 3.2$ currents by $0.1 \mu \mathrm{M}$ UCN (Fig. 6A, left; $I / I_{\text {control }}=0.97 \pm 0.03, n=6$ ). In contrast, the nonhydrolyzable GTP analog guanosine-5'-O-(3-thiotriphosphate) (GTP $\gamma \mathrm{S} ; 100 \mu \mathrm{M}$ ) did not prevent the UCN-mediated inhibition of $\mathrm{Ca}_{\mathrm{V}} 3.2$ currents $\left(I / I_{\text {control }}=0.58 \pm 0.03, n=6, P<\right.$ 0.05 ) and that the inhibition remained for up to 10 min after the washout of UCN. For control cells not dialyzed with intracellular GTP $\gamma \mathrm{S}$, the inhibition by UCN was reversed and the currents returned to baseline levels within 5 min after the washout of UCN (Fig. 6B). These results indicated

that G proteins are involved in the CRFR1-mediated inhibition of $\mathrm{Ca}_{\mathrm{V}} 3.2$ currents. We further determine that which isoform of $\mathrm{G} \alpha$ was involved in this inhibition. To investigate whether the UCN-mediated response occurred via $\mathrm{G} \alpha_{\mathrm{s}}$, we examined its effects on $\mathrm{Ca}_{\mathrm{V}} 3.2$ currents in the presence of CTX, which could catalyze ADP-ribosylation of $\mathrm{G} \alpha_{\mathrm{s}}$. Pretreatment of $\mathrm{Ca}_{\mathbf{V}} 3.2$-expressing cells with CTX ( $500 \mathrm{ng} / \mathrm{ml}$ for 24 h ) abolished the UCN-induced inhibition ( $I / I_{\text {control }}=$ $0.95 \pm 0.06, n=6$ ) (Fig. 6C), indicating that the inhibition of $\mathrm{Ca}_{\mathrm{V}} 3.2$ channels is $\mathrm{G} \alpha_{\mathrm{s}}$-dependent. In addition, after pretreatment of the cells with PTX ( $200 \mathrm{ng} / \mathrm{ml}$ for 24 h ), which catalyzes the ADP-ribosylation of $\mathrm{G} \alpha_{\mathrm{i} / \mathrm{o}}$, UCN still mediated robust inhibition of the $\mathrm{Ca}_{\mathrm{V}} 3.2$ currents $\left(I / I_{\text {control }}=0.67 \pm\right.$ $0.07, n=5$ ) (Fig. 6C). The $\mathrm{Ca}_{\mathrm{V}} 3.2$ currents inhibition induced by CRFR1 activation was sensitive to CTX but not PTX, implicating $\mathrm{G} \alpha_{\mathrm{s}}$, instead of $\mathrm{G} \alpha_{\mathrm{i} / \mathrm{o}}$, was involved. As CTX activated $\mathrm{G} \alpha_{\mathrm{s}}$, we also examined whether CTX would by itself trigger a shift in half-inactivation potential compared with control cells. In CTX-treated cells, we observed a significant shift of $\sim 10 \mathrm{mV}$ in the hyperpolarizing direction of the inactivation potential $(66.7 \pm 0.6$ to $-76.8 \pm 0.9 \mathrm{mV}$ and $k$ from $4.9 \pm 0.6$ to $6.5 \pm 0.8, n=6, P<0.05$ ) (Fig. 6D). To further test whether UCN-mediated $\mathrm{Ca}_{\mathrm{V}} 3.2$ currents inhibition proceeds through a $\mathrm{G} \alpha_{\mathrm{q} / 11}$-mediated pathway, $\mathrm{Ca}_{\mathrm{V}} 3.2$ currents inhibition was examined in the presence of $0.1 \mu \mathrm{~g} / \mu \mathrm{l}$ RGS2, which could selectively bind $\mathrm{G} \alpha_{\mathrm{q} / 11}$-GTP (Kammermeier and Ikeda, 1999). In RGS2-expressing cells, UCN still inhibited
$\mathrm{Ca}_{\mathrm{V}} 3.2$ currents by $\sim 29 \%\left(I / I_{\text {control }}=0.70 \pm 0.02, n=5\right)$ (Fig. 6C), which is not significantly different compared with the RGS2-nontransfected cells. These results suggested that $\mathrm{G} \alpha_{\mathrm{s}}$, not $\mathrm{G} \alpha_{\mathrm{i} / \mathrm{o}}$ and $\mathrm{G} \alpha_{\mathrm{q} / 11}$, is involved in the UCN-induced $\mathrm{Ca}_{\mathrm{v}} 3.2$ channels inhibition.

UCN Inhibited Cave 3.2 Channels Independently of Phospholipase $C$ and Downstream Protein Kinases. Previous reports have shown that cardiovascular and neuronal protections by UCN are mediated via activation of either cAMP/PKA or PKC (Markovic et al., 2006). As it has also been reported that T-type calcium channels can be regulated by serine-threonine kinases, tyrosine kinases (TK), and phospholipase C (PLC) pathways (Chemin et al., 2006), we investigated whether the inhibitory effects of CRFR1 are mediated by these known pathways. After pretreatment of $\mathrm{Ca}_{\mathrm{V}} 3.2$ expressing cells with the selective PKC inhibitors GF109203X $(1 \mu \mathrm{M})$ or chelerythrine chloride $(1 \mu \mathrm{M}), 0.1 \mu \mathrm{M}$ UCN still mediated robust inhibition of the $\mathrm{Ca}_{\mathrm{V}} 3.2$ currents $\left(I / I_{\text {control }}=0.70 \pm 0.04, n=6, P<0.05\right.$ versus control and $I / I_{\text {control }}=0.70 \pm 0.08, n=5, P<0.05$ versus control, respectively) (Fig. 7, A and G). Likewise, pretreatment with selective PLC inhibitors U-73122 (3 $\mu \mathrm{M}$ ) (Suh and Hille, 2002) or ET-18- $\mathrm{OCH}_{3}(10 \mu \mathrm{M})$, did not prevent the inhibition of $\mathrm{Ca}_{\mathbf{V}} 3.2$ currents by $\mathrm{UCN}\left(I / I_{\text {control }}=0.67 \pm 0.03, n=5\right.$, $P<0.05$ versus control and $I / I_{\text {control }}=0.68 \pm 0.05, n=5$, $P<0.05$ versus control, respectively) (Fig. 7, B and G). The degree of inhibition by UCN in the presence or absence of


Fig. 6. G protein-mediated inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.2$ channels by UCN. A, exemplary traces of $\mathrm{Ca}_{\mathrm{v}} 3.2$ currents before ( -UCN ) and during $0.1 \mu \mathrm{M}$ UCN (+UCN) application in the presence of 1 mM GDP $\beta$ S (left). Pooled data show the mean current amplitude $\pm$ S.E.M. of $\mathrm{Ca}_{\mathrm{v}} 3.2$ currents $(n=6$ ). B, time course of UCN inhibition on $\mathrm{Ca}_{\mathrm{v}} 3.2$ currents in absence and presence of GTP $\gamma \mathrm{S}$. Exemplary traces of $\mathrm{Ca}_{\mathrm{v}} 3.2$ currents obtained in presence (left) of GTP $\gamma \mathrm{S}$, before, during, and after washout of the application of $0.1 \mu \mathrm{M}$ UCN. Application of UCN (dark bar, bottom) was for 5 min before washout for the next 10 min (light bar). Both control cells (■) and cells recorded with 0.1 mM GTP $\gamma \mathrm{S}$ in the patch pipette ( $\mathbf{\Delta}$ ) show current inhibition as evaluated at time $=0 \mathrm{~min}$. After washout, $\mathrm{Ca}_{\mathrm{v}} 3.2$ currents recorded in control cells, in the absence of GTP $\gamma \mathrm{S}$, returned to original amplitude, whereas GTP $\gamma$ S-treated cells show a continuous inhibition for up to 10 min . Numbers in parentheses indicate number of cells tested. C, effects of UCN on $\mathrm{Ca}_{\mathrm{v}} 3.2$ currents in PTX ( $200 \mathrm{ng} / \mathrm{ml}$ for 24 h , $n=5$ ) or CTX ( $500 \mathrm{ng} / \mathrm{ml}$ for $24 \mathrm{~h}, n=$ 5)-pretreated cells, and RGS2-expressing HEK293 cells ( $0.1 \mu \mathrm{~g} / \mu \mathrm{l}, n=5$ ). Ca 23.2 currents (A-C) were evoked by a $100-\mathrm{ms}$ depolarizing step to -30 mV from a holding potential of -110 mV . D, effect of $500 \mathrm{ng} / \mathrm{ml}$ CTX on the steady-state inactivation curve of $\mathrm{Ca}_{\mathrm{v}}$ 3.2. Steady-state inactivation curves in the absence ( $\mathbf{-}$ ) or presence ( $\mathbf{\Delta}$ ) of CTX were obtained by evoking $\mathrm{Ca}_{\mathrm{v}} 3.2$ currents with a test depolarization to -30 mV applied at the end of $15-\mathrm{s}$ conditioning pulses ranging from -120 to -30 mV .

PKC inhibitors (GF109203X or chelerythrine chloride) and PLC inhibitors (U-73122 or ET-18-OCH 3 ) were not significantly different. After pretreatment with $100 \mu \mathrm{M}$ genistein, a broad-spectrum inhibitor of TK, and after current amplitudes had stabilized, application of $0.1 \mu \mathrm{M}$ UCN inhibited the $\mathrm{I}_{\mathrm{Ba}}$ by $\sim 27 \%\left(I / I_{\text {control }}=0.73 \pm 0.06, n=5\right)$ (Fig. 7 C ), which was not different from the inhibition obtained in the absence of genistein. Previous reports also showed that CaMKII and phosphatidylinositol 3 -kinase (PI3K) were involved in G protein-mediated regulation of T-type calcium channels (Chemin et al., 2006). Here, preincubation with 1 $\mu \mathrm{M}$ wortmannin, a specific PI3K inhibitor, or $0.5 \mu \mathrm{M}$ KN-93, a selective CaMKII inhibitor, failed to prevent the UCNinduced inhibition of $\mathrm{Ca}_{\mathrm{V}} 3.2$ channel currents $\left[I / I_{\text {control }}=\right.$ $0.69 \pm 0.08, n=5, P<0.05$ versus control) (Fig. 7D); $I / I_{\text {control }}=0.65 \pm 0.06, n=5, P<0.05$ versus control (Fig. 7E)], indicating that the PI3K and CaMKII pathways were not involved. As activation of CRFR1 increases cAMP accumulation and PKA activity (Dautzenberg et al., 2002), blocking PKA activity might be expected to block the effect of

UCN-mediated inhibition. To test this possibility, we dialyzed the cells with a pipette solution containing PKI 5-24 (1 $\mu \mathrm{M})$ and found no effect on the $\mathrm{Ca}_{\mathrm{V}} 3.2$ currents over 5 min . After this pretreatment of PKI $5-24$ for 5 min , application of $0.1 \mu \mathrm{M}$ UCN still reduced current amplitudes by $30 \%$ (I/ $I_{\text {control }}=0.70 \pm 0.05, n=6, P<0.05$ versus control) (Fig. 7F) similar to that for currents recorded in the absence of PKI 5-24. Similar inhibition by UCN on $\mathrm{Ca}_{\mathrm{V}} 3.2$ currents with 0.5 $\mu$ M H-89 $\left(\sim 29 \% ; I / I_{\text {control }}=0.71 \pm 0.07, P<0.05\right.$ versus control, $n=5$ ) was also obtained (Fig. 7G). Taken together, these results indicate that the inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.2$ currents mediated by CRFR1 is not likely to be mediated via PLC or downstream PKA, PKC, CaMKII, PI3K, or TK signaling pathways.

UCN Inhibited Ca $\mathbf{C a}_{\mathbf{v}}$.2 T-Type Calcium Channels via G Protein $\boldsymbol{\beta} \boldsymbol{\gamma}$ Subunits. We further tested whether the UCN-induced inhibition of $\mathrm{Ca}_{\mathrm{V}} 3.2$ currents might be mediated via $\mathrm{G} \beta \gamma$ subunits by introducing into the recording pipette a synthetic peptide, QEHA (encoding residues 956 to 982 of adenylyl cyclase 2), which competitively binds $G \beta \gamma$



D






Fig. 7. UCN inhibits $\mathrm{Ca}_{\mathrm{v}} 3.2$ channels independently of PKC, PKA, TK, PI3K, CaMKII, and PLC (A-G). $\mathrm{Ca}_{\mathrm{V}} 3.2$ currents were evoked by a $100-\mathrm{ms}$ depolarizing step to -30 mV from a holding potential of -110 mV . Cells were incubated with $1 \mu \mathrm{M}$ GF109203X for $30 \mathrm{~min}(\mathrm{~A} ; n=6), 3$ $\mu \mathrm{M} \mathrm{U}-73122$ for $30 \mathrm{~min}(\mathrm{~B} ; n=5)$, $100 \mu \mathrm{M}$ genistein for $30 \mathrm{~min}(\mathrm{C} ; n=$ 5), $1 \mu \mathrm{M}$ wortmannin ( $\mathrm{D} ; n=5$ ), and $0.5 \mu \mathrm{M} \mathrm{KN}-93$ ( E ; $n=5$ ), or intracellular applied $1 \mu \mathrm{M}$ PKI 5-24 (F; $n=6$ ). Pretreatment of cells with 0.5 $\mu$ M H-89 $(n=5), 10 \mu$ M ET-18-OCH 3 (ET; $n=5$ ), and $1 \mu \mathrm{M}$ chelerythrine chloride ( $n=5$ ) is shown (G). The amplitude of the peak $\mathrm{Ca}_{\mathrm{v}} 3.2$ current in the presence of UCN is normalized to the peak current recorded before drug application. Exemplary traces ( $\mathrm{A}-\mathrm{F}$, top) and pooled data ( $\mathrm{A}-\mathrm{F}$, bottom) show $\mathrm{Ca}_{\mathrm{v}} 3.2$ currents in the absence ( -UCN ) or presence ( +UCN ) of $0.1 \mu \mathrm{M} \mathrm{UCN} . *, P<0.05$ versus control.


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and blocks $\mathrm{G} \beta \gamma$-mediated signaling (Chemin et al., 2006). As a control, we used a synthetic SKEE peptide, representing the cognate region of adenylyl cyclase 3, that does not bind G $\beta \gamma$. Our results showed that intracellular pipette application of $200 \mu \mathrm{M}$ QEHA suppressed the effects of $0.1 \mu \mathrm{M} \mathrm{UCN}$ $\left(I / I_{\text {control }}=0.97 \pm 0.03, n=6\right.$ for -UCN and $0.95 \pm 0.04$, $n=9$ for +UCN ) (Fig. 8, A and B). Furthermore, the intracellular application of the QEHA peptide also eliminated the hyperpolarizing shift in the steady-state inactivation potential normally elicited by UCN ( $V_{1 / 2}=-69.6 \pm 0.7$ for control and $V_{1 / 2}=-67.8 \pm 0.9 \mathrm{mV}$ for UCN + QEHA, $n=5, P>$ 0.05 ) (Fig. 8E). In contrast, intracellular application of 200 $\mu \mathrm{M}$ SKEE peptide did not significantly affect the UCN-in-
duced inhibition of $\mathrm{Ca}_{\mathrm{V}} 3.2$ currents $\left(I / I_{\text {control }}=0.97 \pm 0.02\right.$, $n=6$ for -UCN and $0.69 \pm 0.04, n=7$ for +UCN ) (Fig. 8, C and D). Beside intracellular application of QEHA, a mem-brane-associating C-terminal construct of a G protein-coupled receptor kinase that sequesters $\mathrm{G} \beta \gamma$ (MAS-GRK3) was transfected into HEK293 cells to further determine whether $\mathrm{G} \beta \gamma$ is involved in the inhibition of $\mathrm{Ca}_{\mathrm{V}} 3.2$ currents. When MAS-GRK3 was coexpressed in $\mathrm{Ca}_{\mathrm{V}} 3.2$-expressing HEK293 cells, the robust inhibition by UCN was almost eliminated $\left(I / I_{\text {control }}=0.92 \pm 0.09, n=5\right)$ (Fig. 8G). These results demonstrate that $\mathrm{Ca}_{\mathrm{v}} 3.2$ channel inhibition is mediated via a $\mathrm{G} \beta \gamma$-dependent mechanism upon the activation of the CRFR1.


Fig. 8. Evidence that $\mathrm{G} \beta \gamma$ mediates CRFR1-dependent inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.2$ currents. $\mathrm{A}, 200 \mu \mathrm{M}$ synthetic QEHA peptide prevented current inhibition by $0.1 \mu \mathrm{M}$ UCN perfused continuously as indicated by arrow. Inset, without UCN (■) and $0.1 \mu \mathrm{M}$ UCN ( $\mathbf{\Delta}$ ). B, pooled data indicate QEHA peptide abolition of UCN-induced inhibition of $\mathrm{Ca}_{\mathrm{V}} 3.2$ currents in the absence ( $-\mathrm{UCN}, n=6$ ) or presence of $0.1 \mu \mathrm{M} \mathrm{UCN}(+\mathrm{UCN}, n=9)$. C, control $200 \mu \mathrm{M}$ SKEE peptide did not prevent UCN-induced inhibition. Inset, without UCN (■) and + UCN $(0.1 \mu \mathrm{M})(\mathbf{\Delta})$. D, pooled data indicate SKEE peptide effect on UCN-induced inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.2$ currents in the absence ( - UCN, $n=6$ ) and presence of $0.1 \mu \mathrm{M} \mathrm{UCN}(+\mathrm{UCN}, n=7)$; ${ }^{*}, P<$ 0.05 versus control. E , steady-state inactivation curve of QEHA peptide in the absence of UCN $(\square)(n=5)$ and presence of $0.1 \mu \mathrm{M} \mathrm{UCN}(n=5)(\mathbf{\Delta})$. F , left, PCR products of $\mathrm{G}_{\beta 2}(1023$ base pairs) and $\mathrm{G}_{\mathrm{r}_{2}}$ (216 base pairs) amplified from HEK293 mRNA. Negative controls: without adding RT ( -RT ) or template (water). Right, $\mathrm{G}_{\beta 2}$ (top) was expressed in HEK293 cells and rat brain, with $\beta$-actin as control (bottom). G, effects of UCN on $\mathrm{Ca}_{\mathrm{V}} 3.2$ currents in $0.01 \mu \mathrm{~g} / \mu \mathrm{l}$ MAS-GRK3-coexpressed HEK293 cells ( $n=5$ ).

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G protein-dependent inhibition of calcium channels has been previously demonstrated to require the $\mathrm{G}_{\beta 2}$ subunit, whereas the requirement for a specific $\mathrm{G}_{\gamma 2}$ subunit is less stringent (Wolfe et al., 2003). To support our results that the inhibition of $\mathrm{Ca}_{\mathrm{V}} 3.2$ channels induced by CRFR1 activation was via $\mathrm{G} \beta \gamma$, we investigated whether $\mathrm{G}_{\beta 2}$ or $\mathrm{G}_{\gamma 2}$ subunit was endogenously expressed in HEK293 cells. We found by RT-PCR that $\mathrm{G}_{\beta 2}$ and $\mathrm{G}_{\gamma 2}$ transcripts are endogenously expressed in HEK293 cells (Fig. 8F, left). Endogenous $G_{\beta 2}$ protein was also detected by anti- $\mathrm{G}_{\beta 2}$ in HEK293 cells (Fig. 8 F , right). Taken together, these results strongly suggest that native CRFR1 receptors couple via an endogenous G $\beta \gamma-$ dependent signaling pathway to mediate the selective inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.2$ channels among the T-type subfamily of calcium channels. This pathway represents a novel CRFR1coupled mechanism distinct from the previously described PLC and downstream kinase signaling pathways.

## Discussion

CRF peptides mediate multiple physiological responses, including those involved in mammalian responses to stress and inflammation. There is also growing evidence that CRF peptides contribute toward human disorders such as anxiety, depression, and alcohol and drug addictions (Reul and Holsboer, 2002; Bale and Vale, 2004; Gravanis and Margioris, 2005, Bruijnzeel and Gold, 2005). Several CRF receptor subtypes are known to be expressed, and there is considerable interest in determining both the downstream effectors and mechanisms by which these GPCRs mediate their widespread physiological functions. In the present article, we demonstrate that activation of CRFR1 receptors by either CRF or UCN robustly inhibits $\mathrm{Ca}_{\mathrm{V}} 3.2$ channels via a $\mathrm{G} \beta \gamma$ dependent mechanism with no effect on either $\mathrm{Ca}_{\mathrm{v}} 3.1$ or Cave 3.3 T-type calcium channels. We also find that the CRFR1-mediated inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.2$ channels is state-dependent, likely occurring through stabilizing the channels in the inactivated state, and the inhibition is reversible after washout of UCN.
HEK293 cells are an appropriate expression system as these cells endogenously and exclusively express the cortico-tropin-releasing factor receptor 1, the CRFR1 subtype (Dautzenberg et al., 2000) (Fig. 1). Both the activation of endogenous and overexpressed cloned CRFR1 receptors produced robust and selective inhibition of cloned rat and human brain $\mathrm{Ca}_{\mathrm{V}} 3.2$ T-type calcium channels. CRF and UCN have both been shown to bind CRFR1 receptors with similar affinities (Dautzenberg et al., 2000), and we found that $\mathrm{Ca}_{\mathrm{v}} 3.2$ channel inhibition was also not ligand-dependent as both CRF and UCN elicited comparable levels of T-type calcium channels inhibition.
The G protein-dependent inhibition of T-type calcium channels has been previously demonstrated to require the $G_{\beta 2}$ subunit, whereas the requirement for a specific $G_{\gamma}$ subunit is less stringent (Wolfe et al., 2003). These reports have found that inhibition mediated by G protein decreases the probability of opening of the $\mathrm{Ca}_{\mathrm{V}} 3.2$ calcium channels, whereas activation and inactivation properties are unaltered. Note that, in our present findings, the activation of CRFR1 receptors by either CRF or UCN produced a distinct and significant hyperpolarizing shift in the steady-state inactivation potentials, whereas activation potentials re-
mained unaltered. Unlike the activation of the dopamine receptor 1, which requires both cAMP and G protein to act in combination to inhibit T-type calcium currents (Drolet et al., 1997), a similar requirement was not evident for the CRFR1mediated inhibition of the $\mathrm{Ca}_{\mathrm{V}} 3.2$ channels. Evaluating the role of cAMP in CRFR1-mediated inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.2$ channels, application of PKI 5-24, a potent and selective peptide inhibitor of PKA, failed to diminish the inhibitory effects of CRFR1 activation, indicating that PKA is not directly involved in $\mathrm{Ca}_{\mathrm{V}} 3.2$ inhibition.
Previous reports have shown that T-type calcium currents can also be modulated by the PKC signaling pathway. In one instance, examining GH3 cells showed that native T-type calcium currents are inhibited by 1-oleoyl-2-acetylglycerol (a diacylglycerol analog) (Herrington and Lingle, 1992). Similar results were reported for native T-type currents in chick dorsal root ganglion neurons, although in these neurons the direct inhibition was independent of PKC activation (Hockberger et al., 1989). Here, GF109203X, a selective PKC inhibitor, failed to prevent the inhibition by UCN, indicating that PKC is also not involved in $\mathrm{Ca}_{\mathrm{V}} 3.2$ channel inhibition. Previous reports have suggested that PLC activation could also be part of the CRFR1-signaling pathway (Radulovic et al., 2003). PLC is a critical component of the phosphoinositol pathway that generates two second messengers, diacylglycerol and inositol 1,4,5-trisphosphate, that are thought to be involved in some types of T-type calcium channel regulation (Chemin et al., 2006). We found that U-73122, a phospholipase C inhibitor, was unable to abrogate the inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.2$ induced by the UCN-mediated activation of CRFR1 receptor, ruling out the possible involvement of the PLC pathway. Furthermore, the inhibition of $\mathrm{Ca}_{\mathrm{V}} 3.2$ channels via the CRFR1 receptor is independent of the activation of tyrosine kinases, CaMKII, and PI3K, through which T-type calcium channels could be regulated. The explanation for the differences remains to be determined, but it might be attributed to the diversity of UCN activities on cells or that the A-kinase-anchoring protein-deficient HEK293 cells may not be capable of supporting robust ion channel phosphorylation. In addition, it has been reported that kinase modulation of T-type calcium channels has been shown to be temperaturedependent in which phosphorylation was observed at $37^{\circ} \mathrm{C}$, but it was less obvious when the experiments were performed at room temperature (Chemin et al., 2007). These factors may be involved in CRFR1-mediated $\mathrm{Ca}_{\mathrm{v}} 3.2$ regulations, and they need to be further examined.
Native and cloned T-type calcium channel properties have been reported to be regulated by cAMP-dependent PKA, PKC, PKG, calmodulin-dependent protein kinase II, and tyrosine kinases through various G protein-coupled receptors (Herrington and Lingle, 1992; Kim et al., 2006). We find that $\mathrm{G}_{\beta 2}$ and $\mathrm{G}_{\gamma 2}$ transcripts and proteins are endogenously expressed in HEK293 cells, which suggests the potential involvement of $\mathrm{G}_{\beta 2}$ in the selective inhibition of $\mathrm{Ca}_{\mathbf{v}} 3.2$ channels by activation of the CRFR1 receptor. However, other possible subtypes of G protein $\beta \gamma$ subunits could not be excluded, and they need to be further investigated. Previous reports have shown that activation of dopamine D1 and D2 receptors could mediate the T-type calcium channels inhibition (Wolfe et al., 2003; Chemin et al., 2006). Nevertheless, the inhibitory mechanism of $\mathrm{Ca}_{\mathrm{v}} 3.2$ channels mediated by CRFR1 receptor is distinctly different from the activation of

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the dopamine receptors (Wolfe et al., 2003). Our data suggested that the CRFR1-mediated selective inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.2$ was through the stabilization of the channels in their inactivated state, whereas activation of D1 receptors reduces the open probability of the $\mathrm{Ca}_{\mathrm{v}} 3.2$ channels, and the voltagedependent steady-state inactivation of the channels remains unchanged. Evidence to suggest our conclusion includes 1) robust hyperpolarized shift of voltage-dependent steadystate inactivation, 2) reversal of the hyperpolarized shift of inactivation potentials after introduction of the QEHA competitor peptide or the washout of UCN from the bath solution, and 3) a reduction in the concentration of UCN to trigger inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.2$ channels when cells are held at the more depolarized membrane potential of -80 mV compared with that for -110 mV . However, a comparison of the rat $\mathrm{Ca}_{\mathrm{v}} 3.2$ (AF290213) used in this report with the human $\mathrm{Ca}_{\mathrm{v}} 3.2$ channel used by the Barrett's group (AF290213) did not show obvious differences in the choice of alternatively spliced exons. But, the two clones contained differences in amino acid residues that code for the II-III linker. Whether these species differences in the cytoplasmic regions of the channels, including the II-III linker, are important to account for the differences in mechanisms for inhibition by CRFR1 and dopamine receptors require future work. Besides, we used a 15 -s protocol that is different from the 6 -s voltage prepulses used by the Barrett's group (Wolfe et al., 2003). This difference in experimental protocol may be relevant as it has been demonstrated that a shorter duration of the prepulse resulted in a depolarizing shift in the steadystate inactivation in the $\mathrm{G} \beta \gamma$-mediated inhibition of $\mathrm{Ca}_{\mathrm{v}} 2.2$ channels (McDavid and Currie, 2006). Nonetheless, besides the observed hyperpolarized shift in steady-state inactivation, we cannot exclude the possibility that the probability of opening of the $\mathrm{Ca}_{\mathrm{V}} 3.2$ channels may also be reduced upon activation of CRFR1 receptors by UCN. There is a possibility that both mechanisms act as a unified pathway to inhibit the $\mathrm{Ca}_{\mathrm{v}} 3.2$ currents upon activation of CRFR1 receptors by UCN.
In the present study, we found that G protein-mediated $\mathrm{Ca}_{\mathrm{v}} 3.2$ channels inhibition was reversible, which is consistent with the previous reports about $\mathrm{Ca}_{\mathrm{v}} 2$ channels. However, our result showed that the kinetics of $\mathrm{Ca}_{\mathrm{v}} 3.2$ channels inhibition induced by CRFR1 activation seemed to be slow, whereas $\mathrm{G} \beta \gamma$ inhibition of $\mathrm{Ca}_{\mathrm{v}} 2$ channels was very fast ( De Waard et al., 1997). The explanation for the differences remains to be determined, but it might be attributed to channel internalization during the slowly developing process or to the diversity of UCN activities on cells. In addition, whereas G $\beta \gamma$ subunits inhibit high-voltage-gated $\mathrm{Ca}_{\mathbf{v}} 2$ calcium channels via binding to the I-II linker region, they distinctly bind to the II-III linker of $\mathrm{Ca}_{\mathrm{v}} 3.2$ channels (Ikeda, 1996; Zamponi and Snutch, 1998). The binding of the G $\beta \gamma$ subunits to $\mathrm{Ca}_{\mathrm{v}} 2.2$ channels has been reported to alter the voltage-dependent activation or inactivation properties of the channels, depending upon the frequency of the action potential-like waveforms examined (Zamponi and Snutch, 1998). Using low-frequency action potential-like waveforms, $\mathrm{Ca}_{\mathrm{v}} 2.2$ N-type channels display a depolarizing shift in the steadystate inactivation potentials when the conditioning pulse is applied for 3 s (McDavid and Currie, 2006). Given that the $\mathrm{G} \beta \gamma$ effects on $\mathrm{Ca}_{\mathrm{v}} 2.2$ N-type channels can vary depending upon the nature of the stimulus, future studies will be required to dissect the differences in the effects on steady-state
inactivation properties of $\mathrm{Ca}_{\mathrm{V}} 3.2$ channels observed after activation of CRFR1. Recent studies by Iftinca and colleagues have identified a depolarizing shift on $\mathrm{Ca}_{\mathrm{v}} 3.2$, but not $\mathrm{Ca}_{\mathrm{v}} 3.1$ and $\mathrm{Ca}_{\mathrm{v}} 3.3$, inactivation properties in response to Rho-associated kinase activation via the endogenous ligand lysophosphatidic acid (Iftinca et al., 2007). Like G $\beta \gamma$ regulation on $\mathrm{Ca}_{\mathrm{V}} 3.2$ channels, this mechanism involved the II-III loop, and it is tempting to speculate the existence of cross talk between these two regulatory pathways.

In conclusion, the present study provides evidence that activation of CRFR1 results in the selective inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.2$ T-type calcium currents, whereas $\mathrm{Ca}_{\mathrm{v}} 3.1$ and $\mathrm{Ca}_{\mathrm{v}} 3.3$ were not affected. The mechanism for the inhibition was via a cholera toxin-sensitive, $\mathrm{G} \beta \gamma$-mediated signaling pathway that resulted in a hyperpolarized shift in the steady-state inactivation property of the $\mathrm{Ca}_{\mathrm{V}} 3.2$ calcium channels. Note that the effect is reversible upon washout of the CRFR1 receptor agonists such as CRF or UCN. As UCN affect multiple aspects of cardiac and neuronal physiology and as $\mathrm{Ca}_{\mathrm{v}} 3.2$ channels are expressed widely throughout the cardiovascular and nervous systems, our results point to a novel and functionally relevant CRFR1-calcium channel signaling pathway. In future experiments, it will be interesting to determine whether selective inhibition of $\mathrm{Ca}_{\mathrm{v}} 3.2$ calcium channels via activation of CRFR1 receptors may directly play a role in stress response, affective disorders, or in cardiovascular pathophysiology.

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## References

Bale TL and Vale WW (2004) CRF and CRF receptors: role in stress responsivity and other behaviors. Annu Rev Pharmacol Toxicol 44:525-527.
Bourinet E, Alloui A, Monteil A, Barrere C, Couette B, Poirot O, Pages A, McRory J, Snutch TP, Eschalier A, et al. (2005) Silencing of the $\mathrm{Ca}_{\mathrm{v}} 3.2$ T-type calcium channel gene in sensory neurons demonstrates its major role in nociception. EMBO J 24:315-324.
Bruijnzeel AW and Gold MS (2005) The role of corticotrophin-releasing factor peptides in cannabis, nicotine, and alcohol dependence. Brain Res Rev 49:505-528. Chalmers DT, Lovenberg TW, and De Souza EB (1995) Localization of novel corti-cotropin-releasing factor receptor (CRF2) mRNA expression to specific subcortical nuclei in rat brain: comparison with CRF1 receptor mRNA expression. $J$ Neurosci 15:6340-6350.
Chemin J, Traboulsie A, and Lory P (2006) Molecular pathways underlying the modulation of T-type calcium channels by neurotransmitters and hormones. Cell Calcium 40:121-134.
Chemin J, Mezghrani A, Bidaud I, Dupasquier S, Marger F, Barrère C, Nargeot J, and Lory P (2007) Temperature-dependent modulation of Cay 3 T-type calcium channels by protein kinases C and A in mammalian cells. J Biol Chem 282:32710-32718.
Cribbs LL, Lee JH, Yang J, Satin J, Zhang Y, Daud A, Barclay J, Williamson MP, Fox M, Rees M, et al. (1998) Cloning and characterization of alpha 1 H from human heart, a member of the T-type $\mathrm{Ca}^{2+}$ channel gene family. Circ Res 83:103-109.
Dautzenberg FM, Higelin J, and Teichert U (2000) Functional characterization of corticotropin-releasing factor type 1 receptor endogenously expressed in human corticotropin-releasing factor type 1 receptor endogenous
embryonic kidney 293 cells. Eur J Pharmacol 390:51-59.
Dautzenberg FM and Hauger RL (2002) The CRF peptide family and their receptors yet more partners discovered. Trends Pharmacol Sci 23:71-77.
De Waard M, Liu H, Walker D, Scott VE, Gurnett CA, and Campbell KP (1997) Direct binding of G-protein betagamma complex to voltage-dependent calcium channels. Nature 385:446-450.
Drolet P, Bilodeau L, Chorvatova A, Laflamme L, Gallo-Payet N, and Payet MD (1997) Inhibition of the T-type $\mathrm{Ca}^{2+}$ current by the dopamine D1 receptor in rat adrenal glomerulosa cells: requirement of the combined action of the $G$ betagamma protein subunit and cyclic adenosine $3^{\prime}, 5^{\prime}$-monophosphate. Mol Endocrinol 11: 503-514.
Gravanis A and Margioris AN (2005) The corticotrophin-releasing factor (CRF) family of neuropeptides in inflammation: potential therapeutic applications. Curr Med Chem 12:1503-1512
Herrington J and Lingle CJ (1992) Kinetic and pharmacological properties of low voltage-activated $\mathrm{Ca}^{2+}$ current in rat clonal (GH3) pituitary cells. J Neurophysiol 68:213-232.

## Appendix 2: Activation of CRFR1 selectively inhibits Cav 3.2

Hildebrand ME and Snutch TP (2006) Contributions of T-type calcium channels to the pathophysiology of pain signaling. Drug Discov Today Dis Mech 3:335-341.
Hockberger P, Toselli M, Swandulla D, and Lux HD (1989) A diacylglycerol analogue reduces neuronal calcium currents independently of protein kinase C activation Nature 338:340-342.
Iftinca M, Hamid J, Chen L, Varela D, Tadayonnejad R, Altier C, Turner RW, and Zamponi GW (2007) Regulation of T-type calcium channels by Rho-associated kinase. Nat Neurosci 10:854-860.
Ikeda SR (1996) Voltage-dependent modulation of N-type calcium channels by G-protein $\beta \gamma$ subunits. Nature 380:255-258.
Jagannathan S, Punt EL, Gu Y, Arnoult C, Sakkas D, Barratt CL, and Publicover SJ (2002) Identification and localization of T-type voltage-operated calcium channel subunits in human male germ cells. J Biol Chem 277:8449-8456.
Kammermeier PJ and Ikeda SR (1999) Expression of RGS2 alters the coupling of metabotropic glutamate receptor 1 a to M -type $\mathrm{K}^{+}$and N -type $\mathrm{Ca}^{2+}$ channels. Neuron 22:819-829.
Kim JA, Park JY, Kang HW, Huh SU, Jeong SW, and Lee JH (2006) Augmentation of $\mathrm{Ca}_{\mathrm{V}} 3.2$ T-type calcium channel activity by cAMP-dependent protein kinase A. J Pharmacol Exp Ther 318:230-237
Kim Y, Park MK, Uhm DY, and Chung S (2007) Modulation of T-type $\mathrm{Ca}^{2+}$ channels by corticotropin-releasing factor through protein kinase C pathway in MN9D dopaminergic cells. Biochem Biophys Res Commun 358:796-801.
Lee AK and Tse A (1997) Mechanism underlying corticotropin-releasing hormone (CRH) triggered cytosolic $\mathrm{Ca}^{2+}$ rise in identified rat corticotrophs. J Physiol 504:367-378.
Markovic D, Papadopoulou N, Teli T, Randeva H, Levine MA, Hillhouse EW, and Grammatopoulos DK (2006) Differential responses of corticotropin-releasing hormone receptor type 1 variants to protein kinase C phosphorylation. J Pharmacol Exp Ther 319:1032-1042.
McDavid S and Currie KP (2006) G-proteins modulate cumulative inactivation of N-type (Ca 2.2 ) calcium channels. J Neurosci 26:13373-13383.
McKay BE, McRory JE, Molineux ML, Hamid J, Snutch TP, Zamponi GW, and Turner RW (2006) Cav3 T-type calcium channel isoforms differentially distribute to somatic and dendritic compartments in rat central neurons. Eur J Neurosci 24:2581-2594
McRory JE, Santi CM, Hamming KS, Mezeyova J, Sutton KG, Baillie DL, Stea A, and Snutch TP (2001) Molecular and functional characterization of a family of rat brain T-type calcium channels. J Biol Chem 276:3999-4011.

Molineux ML, McRory JE, McKay BE, Hamid J, Mehaffey WH, Rehak R, Snutch TP, Zamponi GW, and Turner RW (2006) Specific T-type calcium channel isoforms are associated with distinct burst phenotypes in deep cerebellar nuclear neurons. Proc Natl Acad Sci U S A 103:5555-5560.
Nelson MT, Todorovic SM, and Perez-Reyes E (2006) The role of T-type calcium channels in epilepsy and pain. Curr Pharm Des 12:2189-2197.
Nuss HB and Houser SR (1993) T-type $\mathrm{Ca}^{2+}$ current is expressed in hypertrophied adult feline left ventricular myocytes. Circ Res 73:777-782.
Perez-Reyes E (2003) Molecular physiology of low-voltage-activated T-type calcium channels. Physiol Rev 83:117-161.
Radulovic M, Hippel C, and Spiess J (2003) Corticotropin-releasing factor (CRF) rapidly suppresses apoptosis by acting upstream of the activation of caspases. $J$ Neurochem 84:1074-1085.
Reul JM and Holsboer F (2002) Corticotropin-releasing factor receptors 1 and 2 in anxiety and depression. Curr Opin Pharmacol 2:23-33.
Steriade M and Llinas RR (1988) The functional states of the thalamus and the associated neuronal interplay. Physiol Rev 68:649-742.
Suh BC and Hille B (2002) Recovery from muscarinic modulation of M current channels requires phosphatidylinositol 4,5-bisphosphate synthesis. Neuron 35: channels
Talley EM, Cribbs LL, Lee JH, Daud A, Perez-Reyes E, and Bayliss DA (1999) Differential distribution of three members of a gene family encoding low voltageactivated (T-type) calcium channels. J Neurosci 19:1895-1911.
Tao J, Wu Y, Chen J, Zhu H, and Li S (2005) Effects of urocortin on T-type calcium currents in mouse spermatogenic cells. Biochem Biophys Res Commun 329:743748.

Welsby PJ, Wang H, Wolfe JT, Colbran RJ, Johnson ML, and Barrett PQ (2003) A mechanism for the direct regulation of T-type calcium channels by $\mathrm{Ca}^{2+} /$ calmodulin-dependent kinase II. J Neurosci 23:10116-10121.
Wolfe JT, Wang H, Howard J, Garrison JC, and Barrett PQ (2003) T-type calcium channel regulation by specific G-protein $\beta \gamma$ subunits. Nature 424:209-213.
Zamponi GW and Snutch TP (1998) Modulation of voltage-dependent calcium channels by G-proteins. Curr Opin Neurobiol 8:351-356.

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## APPENDIX 3: OTHER PHD PUBLICATIONS

Besides my above publications that relate directly to T-type modulation and physiology, I also have contributed to several other publications during my PhD studies (see below). Most significantly, I designed and performed all electrophysiology experiments and participated in the writing of the paper by Spacey et al. (2004). This paper examined the functional impact of a missense mutation in the P/Q-type $\mathrm{Ca}_{\mathrm{v}} 2.1$ channel that was identified in a patient with type-2 episodic ataxia. The H1736L mutation caused a significant reduction in $\mathrm{Ca}_{\mathrm{v}} 2.1$ current density, an increase in inactivation kinetics, and a depolarizing shift in the voltage-dependence of activation, consistent with the loss of P/Q-type channel function that is hypothesized to underlie this form of ataxia. I also performed all experiments and data analysis and participated in the writing of Hildebrand et al. (2004). This study demonstrated that a commonly used househould insecticide (allethrin) potently blocked all major subfamilies of $\mathrm{Ca}^{2+}$ channels $\left(\mathrm{Ca}_{\mathrm{v}} 1.2\right.$, $\mathrm{Ca}_{\mathrm{v}} 2.1$, and $\mathrm{Ca}_{\mathrm{v}} 3.1$ ) by accelerating their inactivation kinetics and shifting their voltage-dependence of inactivation to more hyperpolarized potentials. As the physiological effects of pyrethroid pesticides like allethrin are thought to be mediated by the prolonged opening of voltage-gated $\mathrm{Na}^{+}$channels, our study identified voltage-gated $\mathrm{Ca}^{2+}$ channels as a novel molecular target for pyrethroid action.

## Additional publications during my PhD studies:

Spacey, S.D., Hildebrand, M.E., Materek, L.A., Bird, T.D., and Snutch, T.P. (2004). Functional implications of a novel EA2 mutation in the P/Q-type calcium channel. Ann Neurol 56, 213-220.

Hildebrand, M.E., McRory, J.E., Snutch, T.P., and Stea, A. (2004). Mammalian voltage-gated calcium channels are potently blocked by the pyrethroid insecticide allethrin. J Pharmacol Exp Ther 308, 805813.

McRory, J.E., Hamid, J., Doering, C.J., Garcia, E., Parker, R., Hamming, K., Chen, L., Hildebrand, M., Beedle, A.M., Feldcamp, L., et al. (2004). The CACNA1F gene encodes an L-type calcium channel with unique biophysical properties and tissue distribution. J Neurosci 24, 1707-1718.

Vieira, L.B., Kushmerick, C., Hildebrand, M.E., Garcia, E., Stea, A., Cordeiro, M.N., Richardson, M., Gomez, M.V., and Snutch, T.P. (2005). Inhibition of high voltage-activated calcium channels by spider toxin PnTx3-6. J Pharmacol Exp Ther 314, 1370-1377.

## APPENDIX 4: UBC RESEARCH CERTIFICATES OF APPROVAL

THE UNIVERSITY OF BRITISH COLUMBIA

ANIMAL CARE CERTIFICATE

Application Number: A08-0005
Investigator or Course Director: Terry P. Snutch
Department: Zoology
Animals:

Rats Wistar 250

Start Date: January 1, 2008
Approval Date: January 28, 2008
Funding Sources:

Funding Agency: Canadian Institutes of Health Research (CIHR)
Funding Title: $\quad$ Structure \& function of neuronal calcium channels

Unfunded title: N/A

The Animal Care Committee has examined and approved the use of animals for the above experimental project.
This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

Office of Research Services and Administration
102, 6190 Agronomy Road, Vancouver, BC V6T 1 Z3
Phone: 604-827-5111 Fax: 604-822-5093

# Biohazard Approval Certificate 

```
PROTOCOL NUMBER: H05-0170
INVESTIGATOR OR COURSE DIRECTOR: Snutch,Terrance
DEPARTMENT: Michael Smith Laboratories
PROJECT OR COURSE TITLE: Structure and Function of Neuronal Calcium
Channels
APPROVAL DATE: 07-12-14
APPROVED CONTAINMENT LEVEL: }
FUNDING AGENCY: Canadian Institutes of Health Research (CIHR)
```

The Principal Investigator/Course Director is responsible for ensuring that all research or course work involving biological hazards is conducted in accordance with the Health Canada, Laboratory Biosafety Guidelines, (2nd Edition 1996). Copies of the Guidelines (1996) are available through the Biosafety Office, Department of Health, Safety and Environment, Room 50-2075 Wesbrook Mall, UBC, Vancouver, BC, V6T 1Z1, 822-7596, Fax: 822-6650.

Approval of the UBC Biohazards Committee by one of: Chair, Biosafety Committee
Manager, Biosafety Ethics
Director, Office of Research Services

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required.

A copy of this certificate must be displayed in your facility.


[^0]:    * A version of this chapter has been published. Hildebrand, M.E., David, L.S., Hamid, J., Mulatz, K., Garcia, E., Zamponi, G.W., and Snutch, T.P. (2007). Selective inhibition of Cav3.3 T-type calcium channels by Galphaq/11coupled muscarinic acetylcholine receptors. J Biol Chem 282, 21043-21055.

[^1]:    * A version of this chapter has been submitted for publication. Hildebrand, M.E., Isope, P., Garcia, E., Feltz, A., Schneider, T., Hescheler, J., Kano, M., Sakimura, K., Dieudonne, S., Snutch, T.P. (2008) Functional coupling between mGluR1 and $\mathrm{Ca}_{\mathrm{v}} 3.1$ T-type calcium channels enhances cerebellar Purkinje cell excitability and local signaling. Neuron.

[^2]:    *Corresponding author: T.P. Snutch (snutch@msl.ubc.ca)

[^3]:    * A version of this appendix has been published. Hildebrand, M.E., and Snutch, T.P. (2006). Contributions of Ttype calcium channels to the pathophysiology of pain signaling. Drug Discovery Today: Disease Mechanisms 3, 335-341.

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    doi: $10.1124 / \mathrm{mol} .107 .043612$
    [S The online version of this article (available at http://molpharm. aspetjournals.org) contains supplemental material.

[^5]:    * A version of this appendix has been published. Tao, J., Hildebrand, M.E., Liao, P., Liang, M.C., Tan, G., Li, S., Snutch, T.P., and Soong, T.W. (2008). Activation of corticotropin-releasing factor receptor 1 selectively inhibits $\mathrm{Ca}_{\mathrm{V} 3.2}$ T-type calcium channels. Mol Pharmacol. 73, 1596-1609. Reprinted with permission of the American Society for Pharmacology and Experimental Therapeutics. All rights reserved.

