Identification of a Novel Protein Interaction Which Contributes to N-type Calcium Channel G-protein and PKC-dependent Modulation

by

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Abstract

The N-type calcium channel plays a critical role in neurotransmission and is known to associate with proteins involved in vesicle release such as syntaxin, synaptotagmin and SNAP-25. In addition, block of N-type channels has been shown to abolish neurotransmission in both the peripheral and central nervous systems. Modulation of calcium channels can alter calcium entry into a cell and therefore understanding these processes is crucial towards determining the factors governing N-type channel regulation of neurotransmission.

In order to identify factors which may influence the activity of the N-type channels in neuronal physiology, I screened a rat brain yeast two-hybrid library with the cytoplasmic regions of the rat N-type α_{1B} subunit. Using this approach clones encoding the protein Nell2 were identified that bound to the C-terminus of the N-type α_{1B} subunit. Nell2 is a PKC binding protein and a member of a family of proteins known as Nels (**n**euronally expressed proteins containing **E**GF-**l**ike repeats).

RT-PCR of various rat tissues determined that Nell2 and N-type calcium channel RNA were expressed in many of the same tissues, including brain stem, cerebellum, cortex and pituitary with trace amounts in eye and heart. A full-length Nell2 clone was generated from rat brain RNA by RT-PCR with and without an N-terminal Flag epitope tag. Co-expression of Nell2 and N-type calcium channels was analyzed in HEK cells and neurons by immunohistochemical techniques. Both proteins were found to be membrane associated and localized to the same regions within each cell type.

In order to understand the functional significance of the Nell2/N-type channel interaction,

electrophysiological analysis was carried out on HEK cells co-expressing Nell2 and N-type channels. The presence of Nell2 enhanced both the PKC and G-protein-dependent modulation of N-type channels. In the presence of Nell2, N-type channels became more sensitive to phorbol esters and co-expression with Nell2 antisense RNA abolished the characteristic PKC-dependent upregulation of N-type channel peak currents. The G-protein-dependent inhibition of N-type channel currents was further enhanced in the presence of Nell2. Deletion analysis of the Nell2 gene product showed that the first 20 amino acids largely contributed to the enhanced G-protein-dependent inhibition. A model is proposed to describe how Nell2 may participate in neurotransmission by anchoring PKC at presynaptic sites with the N-type calcium channels.

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List of Abbreviations

AKAP	protein kinase A anchoring protein
Ba^{2+}	barium
BAPTA	1,2-bis(2-aminophenoxy)ethane N,N,N',N'-tetraacetate
bp	base pairs
Ca^{2+}	calcium
Cd^{2+}	cadmium
CNS	central nervous system
Csps	cystein string proteins
DHP	dihydropyridine
DNA	deoxynucleic acid
dNTPs	deoxyribonucleoside triphosphates
DTT	dithiothreitol
EDTA	disodium ethylenediaminetetraacetate
EGTA	ethylene glycol-bis(aminoethylether)N,N,N',N;-tetraacetate
EtBr	ethidium bromide
G Protein	heterotrimeric guanyl-nucleotide-binding-proteins
GABA	γ-aminobutyric acid
GST	glutathione S-transferase
HEK	human embryonic kidney
HVA	high voltage-activated
IPTG	isopropylthio-β-Dgalactoside
kb	kilobase
LVA	low voltage-activated

ml	milliliter
mM	millimolar
ms	millisecond
mV	millivolt
mΩ	mega ohms
MT-PBS	mouse tonicity-phosphate buffered saline
Na^+	sodium
ng	nanogram
Nel	neuronally expressed protein containing EGF-like repeats
Ni^{2+}	nickel
ω-Aga	ω-agatoxin
ω-CTx	ω-conotoxin
ω-CgTx	ω-conotoxin GVIA
pA	picoAmps
PDZ	post synaptic density protein, Drosophila discs large, zonula occludens
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PKA	cAMP-dependent protein kinase
PKC	protein kinase C
PMA	phorbol ester 12-myristate-13-acetate
PMSF	phenylmethylsulfonylflouride
PNS	peripheral nervous system
pS	pico seimens

PTX	pertussis toxin
RNA	ribonucleic acid
rpm	revolutions per minute
RT-PCR	reverse transcription-polymerase chain reaction
SDS	sodium dodecyl sulfate
SNAP-25	synaptosomal protein of 25 kDa
SNARE	soluble N-ethylmaleimide-sensitive attachment factor receptor proteins
SXN	syntaxin
SYN	synaptotagmin
TBS	tris-buffered saline
TEA	tetraethylammonium
τ	time constant
UBC	University of British Columbia
µl	microliter
µg	microgram
VGCC	voltage-gated calcium channel

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Dedication

This thesis is dedicated to my mother Carmen Ann Elizabeth Guthrie, whose unconditional love has given me the courage and strength to succeed.

Chapter 1. Introduction

The Action Potential

The human brain has an estimated 10^{11} neurons classified into over 1000 different types, yet neurons have the same basic architecture and similar properties. The key of perceiving and discriminating between enormous amounts of information and responding appropriately is the result of cellular organization such that similar cells can function differently due to their connective networks within the brain. Cell-cell communication was first demonstrated in the early 19th century when physiologists discovered that neurons are electrically excitable and can affect adjacent cells through the transmission of nerve impulses (Hille, 1992). More recent research on ion channels has been partly responsible for discovering the molecular determinants of this complex communication system.

The "action potential" is the signalling mechanism by which neurons conduct information from one place to another (Kandel, 1991). The action potential was first studied using voltage-clamp techniques by Alan Hodgkin and Andrew Huxley in the 1950s where the membrane was clamped at a specific voltage so that opening and closing of ion channels could be studied without influencing the membrane potential. Eventually, Hodgkin and Huxley presented a model describing channel openings and the propagation of the action potential physiologically in a giant squid axon, revolutionizing the study of excitable cells.

Figure 1A describes the different configurations of the patch clamp technique which developed by Kenneth Cole in 1949 and widely used by electrophysiologists today (Kandel, 1991). Formation of a gigaseal with a glass pipette carrying specific internal solutions allows the experimenter to manipulate the cell and record currents. Set up can vary between cell-attached patch, inside-out patch, whole-cell recording or outside-out patch. **Figure 1B** and **C** shows different types of output that can be generated from the patch clamp technique, single channel currents recorded by cell-attached patch (**B**) or whole-cell currents recorded where the membrane is broken by the pipette and all channels in the cell are observed (**C**).

Using this technique to study the action potential, the flow of ions was measured as current (I) and were separated into the components of sodium, potassium and calcium currents. The ease of ionic flow between two points is the conductance (g) and was examined for each of the different ions. It was later determined that different ion channels were responsible for the changes in membrane potential due to selective permeabilities for specific ions. These channels were acting in a voltage-dependent manner. Current-voltage relations were determined for each channel type involved in an action potential leading to conductance changes at different voltages over time.

Figure 2A shows the potential changes (mV) of a neuron during an action potential and **Figure 2B** shows the conductances (g) involved. Both electrical and chemical gradients are driving an action potential. A chemical gradient exists based on the difference in ion concentrations across the membrane. The electrical potential is determined by the equilibrium potential for a particular ion and can be described mathematically using the Nernst equation. The electrical potential for a particular ion is greatest as the membrane potential moves away from the equilibrium potential of that ion. Initially, a depoloarization in membrane potential causes activation of voltage-gated sodium channels leading to channel opening. The electrical and chemical gradients established

Figure 1. Patch-clamp Configurations.

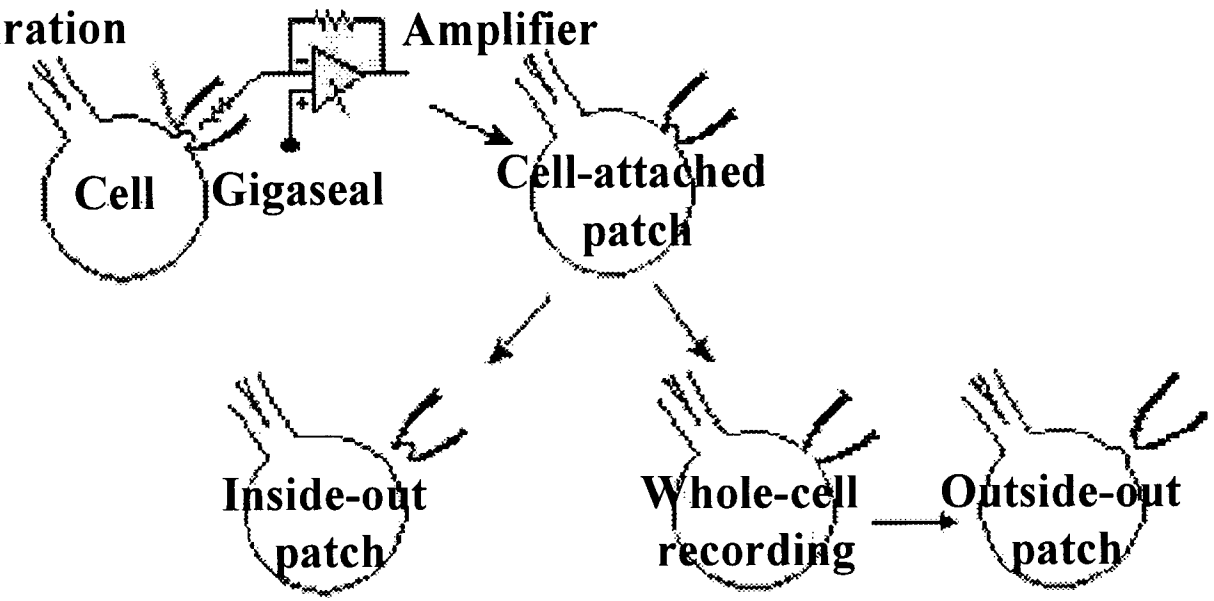
A. Single-channel and whole cell recordings using patch-clamp technique can be used in four different configurations. These include cell-attached patch, inside-out patch, whole-cell recording or outside out patch. Single-channel recordings use all set-ups except whole-cell. Whole-cell recordings are carried out by breaking the gigaseal formed initially by a patch to study whole-cell voltage or current recordings.

B. Single channel recordings from a cultured CNS neuron by cell-attached patch recordings. Upward peaks represent channel openings and at least two channels are present in this patch which is determined by appearance of different amplitudes.

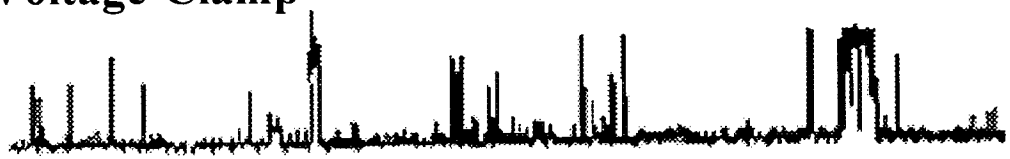
C. Whole-cell recordings on hippocampal neuron in a slice preparation where sodium and potassium channels were blocked (QX-314, 140 mM CsCl, respectively) and a depolarizing current was injected. I = current, V = voltage.

(http://www.acnp.org/G4/GN401000005/5F2.htm).

A. Patch Configuration



B. Single Channel Voltage-Clamp



C. Whole-Cell Current-Clamp

across the plasma membrane for sodium cause rapid sodium influx and trigger further membrane depolarization. **Figure 2A** (point number 1) indicates that the membrane potential has changed from a resting potential of -80 mV to a positive potential. As membrane potential of a cell nears the equilibrium potential for sodium, the chemical and electrical gradients are equal.

Voltage-gated potassium channels are also sensitive to changes in membrane potential, these channels are activated at depolarized potentials and play an important role in the action potential. The result of sodium influx drives the membrane potential from resting to reach the sodium equilibrium potential. The equilibrium potential for potassium causes a huge electrical force for potassium ions (electrical gradient). Since potassium has a large concentration gradient favoring movement of potassium ions out of the cell, potassium channel opening in response to voltage causes a net flow of potassium out of the cell. The efflux of potassium ions results in cell membrane repolarization, restoring the resting potential (**Figure 2B**).

Compared to sodium channels, voltage-gated calcium channels have a delayed response to voltage changes due to slower activation kinetics. These channels open as the membrane potential becomes depolarized from the negative resting potential and also participates in an action potential. As voltage-gated calcium channels open, the electrical/chemical forces for calcium ions cause calcium to flow into the cell. The action potential is propagated along a neuron from the dendrite or cell body to the synaptic terminal, although the synaptic terminal can also recieve input from neighbouring cells and generate an action potential. Neuronal communication results in membrane depolarization of the presynaptic terminal. When the local internal calcium concentration changes from approximately 100 nM to greater than approximately 100 μM during an action potential, neurotransmitter release occurs from presynaptic active zones (Barrett and Stevens, 1972, Kandel, 1991, Augustine and Neher, 1992,

Zucker, 1993). Influx of calcium can also act on calcium-activated potassium channels and inactivate voltage-gated calcium channels participating in cellular repolarization following the action potential.

Calcium channels share many properties with other voltage-gated ion channels. The question of what constitutes a calcium channel, the nature of their composition and how they function can be addressed by studying channels in their native state. While first identified in crustacean muscle (Mounier and Vassort, 1975), calcium currents have been found in all excitable cells of vertebrates, and invertebrates including *C. elegans* (Schafer and Kenyon, 1995) and *Drosophila melanogastar* (Zheng et al., 1995, Smith et al., 1996) as well as yeast (Tettelin et al., 1997) and plants (Reiss and Herth, 1985).

Native Voltage-gated Calcium Channels

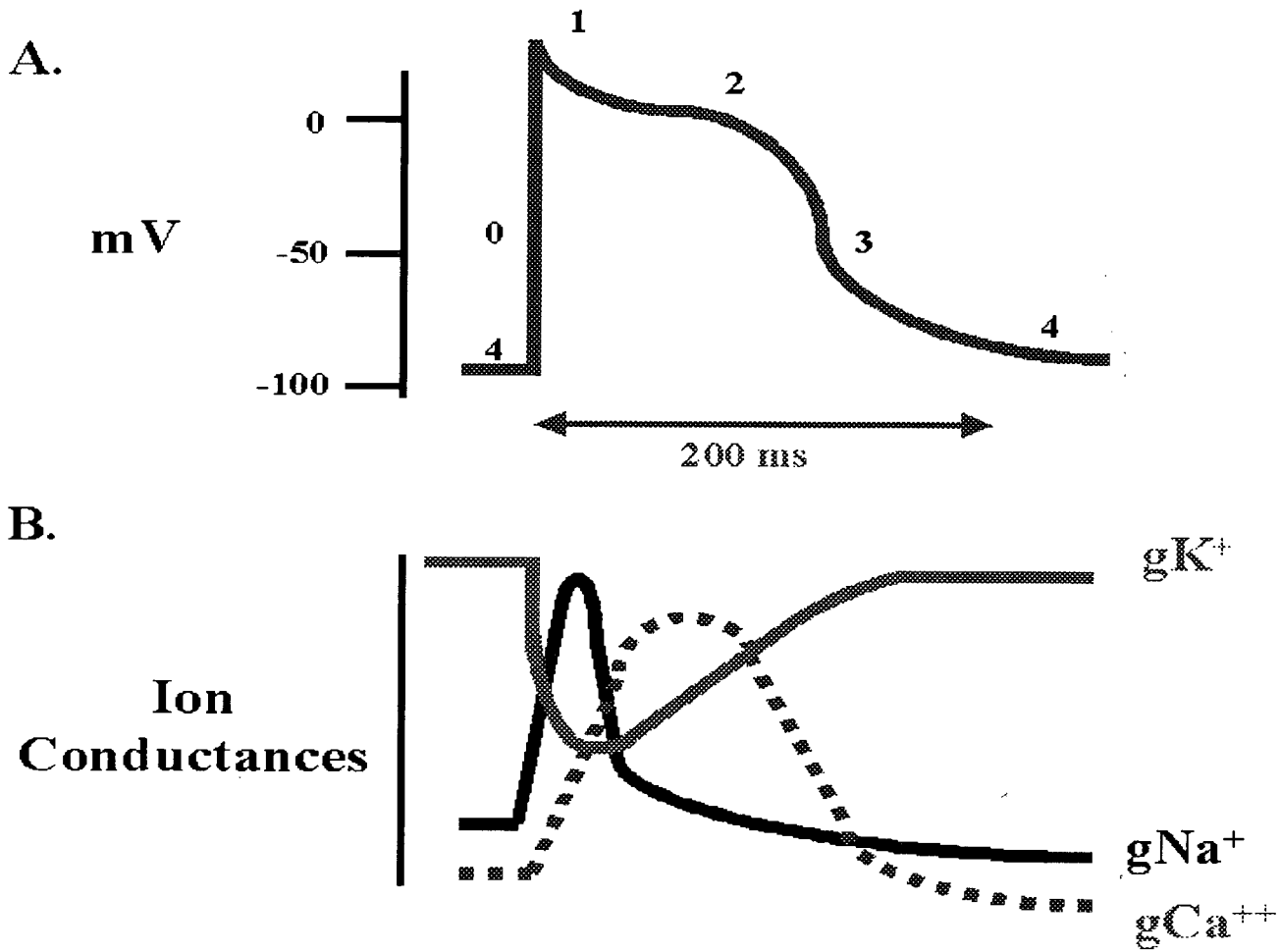
Calcium channels were originally challenging to study since they are relatively small currents, are expressed at low density in native cells and in that calcium influx into cells often changes other currents in the cell. Individual ion channel properties were first studied by patch-clamp technique, a refinement of the voltage-clamp (**Figure 1A**). Isolation of calcium currents in whole cell patch-clamp preparations are done by blocking sodium currents with tetrodotoxin (TTX) and potassium currents by Cs^+ and tetraethylammonium ion (TEA) in the pipette solution.

The first study of calcium channels in mammalian chromaffin cells of the adrenal medulla was done by successive membrane depolarizations which resulted in an increase in channel opening and an increase in inward calcium current (Hagiwara, 1983). When compared to sodium channels, the calcium channels required more membrane depolarization to open and had lower density. It was later determined that calcium channel currents had two components; those

Figure 2. The Action Potential.

A. Membrane potential changes over time during an action potential and has been broken down into five phases (0 - 4). Initially, a cell maintains a negative resting potential (Phase 0). An action potential causes a rapid membrane depolarization (Phase 1) which is delayed (Phase 2) and finally the resting membrane potential is restored (Phase 3 and 4). These events occur at a fast rate, 200 ms or less. It has been determined that the ionic flow of sodium, potasium and calcium are responsible for the characteristic changes in membrane potential during an action potential.

B. Ion conductances from potassium (g_{K+}), sodium (g_{Na+}) and calcium (g_{Ca++}) during an action potential. The initiation of an action potential occurs with the influx of sodium ions by the opening of voltage-gated sodium channels. The opening of voltage-gated potasium channels follows, whereby potassium ions flow out of the cell. Sodium channels quickly inactivate and movement of sodium ions into the cell ceases. Voltage-gated calcium channels have a delay in opening due to slow activation kinetics. Calcium ion influx into the cell prolongs the time a cell remains depolarized during the action potential. Eventually, the potassium and calcium channels inactivate and the resting membrane potential is restored (http://www.oucom.ohiou.edu/CVPhysiology/A006.htm). The membrane potential and conductance differences were determined by recordings from the giant axon of the squid (Kandel, 1991).


A.
mV
0
-50
-100
1
2
3
4
0
4
200 ms
B.
Ion
Conductances
gK+
gNa+
gCa++

channels which opened close to the resting cell membrane potential, called low voltage-activated (LVA), and those channels that opened at high voltages (>-70 mV), classified as high voltage-activated (HVA). Different classes of calcium channels coexist within the same cell and have different sensitivities to small depolarizations.

Although calcium was shown to be involved in muscle contraction, ion channel gating and as a second messenger within cells, it was particularly interesting to correlate neurotransmitter release as a calcium-dependent process in neurons. When an action potential reaches the nerve terminal, vesicles filled with neurotransmitter fuse and release their contents by calcium-dependent exocytosis. Study of this process was best observed optically in the squid giant synapse where fluorescent calcium dyes were used as inward current was controlled by voltage clamp. As with secretion of hormones, digestive enzymes, cortical granules, acrosomes and mucus from many other cell types, calcium was required for secretion of neurotransmitters in nerve terminals.

In order to discern the role of calcium channels in neurotransmission, one must realize the diversity of channel function at the physiological level. The muscle contraction of the heart requires oscillary calcium spikes while others, such as those from endocrine cells, are long lasting and continuous, requiring the maintenance of calcium influx into a cell. Calcium channels are not the only source of increasing internal calcium within the cell and it was already clear that more than one channel type must exist to provide the LVA and HVA currents. Thus distinguishing a family of proteins of calcium channels and mechanisms of modulation of those channels may reveal how multiple secretory events can occur to provide cellular communication.

To date, five distinct types of calcium channels have been described in native cells and have been classified by time and voltage-dependence of inactivation, conductance, permeability, open

and close time duration and pharmacological properties (T-, L-, N-, P-, Q- and R-types) (Nowycky et al., 1985, Miller, 1987, Tsien et al., 1988, Bean, 1989, Hess, 1990, Scott et al., 1991, Randall, 1993, Wheeler et al., 1994, **see Table 1**). The HVA channels include N-, L-, P/Q- and R-type channels which first activate at more positive potentials. LVA channels are composed of the T-type channels, first activating at relatively negative potentials (Bean, 1989, Akaike, 1991, Tsien et al., 1991, Bean and McDonough, 1998, Huguenard, 1998, Perez-Reyes, 1998, Perez-Reyes et al., 1998). There remain some calcium currents that do not fit neatly into either category suggesting that the distinction between HVA and LVA channels may be somewhat artificial (Artalejo et al., 1992, Elmslie et al., 1994, Forti et al., 1994). The initial nomenclature was introduced in 1985 and has further developed as additional cell types and pharmacological agents have been examined (Nowycky et al., 1985, Fox et al., 1987a and Fox et al., 1987b).

Voltage-gated calcium channels can also be distinguished based on sensitivity to various calcium channel blockers, dihydropyridines (DHPs), mollusk *Conus geographus* peptide toxin (ω-conotoxin GVIA) and a peptide from the funnel web spider *Agelenopsis aperta* (ω-Aga IVA). All calcium channel currents are also blocked by inorganic divalent cations such as Cd^{2+}, Ni^{2+} and Co^{2+} (Dolphin, 1995). Internal Mg^{2+} can also block these channels. L-type requires 1 mM and N-type channels are more sensitive at 200 μM.

The L-type Channels

L-type channels were first described in chick dorsal root ganglia and originally named "L" for their long lasting activation with Ba^{2+} as the charge carier and their large single-channel conductance (Nowycky et al., 1985). L-type channels have since been described in skeletal, cardiac and smooth muscle as well as brain tissue. Using Ba^{2+} as the charge carrier, the L-type channel conductance has been found to be 11 to 25 pS. The channel first activates around –30

Table 1. Properties of Native Calcium Channels.

Native voltage-dependent calcium channels have been classified both biophysically using electrophysiology recordings and pharmacologically using calcium channel antagonists. Table 1 summarizes data collected from various groups and reviewed by Dolphin, 1995, using whole cell and single channel recordings with Ba^{2+}. The values are approximated and vary according to species, cell type, charge carrier and conditions used for recordings. HVA, high voltage activated, LVA, low voltage activated, pS, picosiemens, mV, millivolts, msec, milliseconds, V_{50}, membrane potential when half channels are activated.

Class	Type	Conductance (~ pS)	Activ-ation (~ >mV)	Steady-state Inactivation V_{50} (~ mV)	Inactivation Rate (~ msec)	Localization
L	HVA	11-25 (Ba2+>Ca2+)	-30	-60 to –10	>500	Neuronal, endocrine, cardiac, smooth, and skeletal muscle, fibroblasts cells, kidney cells and retina
N	HVA	10-22 (Ba2+>Ca2+)	-30	-120 to –30	50 to >500	Neurons, endocrine cells, eye, kidney and liver
P/Q	HVA	9-19	-40	-45 to -5	>500	Purkinje cells
R	HVA	14 (Ba2+>Ca2+)	-40	-100 to –15	20 to 40	Cerrebellar granule cells
T	LVA	7-10 (Ba2+=Ca2+)	-70	-110 to -50	14 to 80	Neuronal tissue, smooth muscle, cardiac and skeletal muscle

mV and has an inactivation (V_{50}) range from –60 to -10 mV with an inactivation rate slower than 500 msec. The skeletal muscle L-type channel has lower conductance and activates slower than the other L-type channels. In contrast to that for Ba^{2+}, when calcium ions are used as the charge carrier, L-type channel currents are smaller and inactivate faster due to calcium-dependent inactivation.

Calcium-dependent inactivation has been characterized by rapidly decaying calcium currents while longer lasting currents exist when Ba^{2+}, strontium (Sr^{2+}) and Na^{+} ions are used as the charge carrier. Inactivation peaks at membrane potentials where calcium influx is maximal and calcium chelators (BABTA) can slow this degree of inactivation. Application of trypsin abolishes inactivation and had led to the hypothesis that calcium inactivates the channel by a intrinsic or close to the channel mechanism (Bean, 1989a; Scott et al., 1991, Charnet et al., 1994; Neely et al., 1994). Several mechanisms have since been proposed to describe L-type channel calcium-dependent inactivation (Chad and Eckert, 1986, Scott et al., 1991, Imredy and Yue, 1994).

In 1986 Chad and Eckert described a model whereby second messengers are activated by calcium influx leading to dephosphorylation events resulting in inactivation of the channel. Candidate molecules included calcineurin and calmodulin. However, when phosphatase and calmodulin activity was inhibited and calcium-dependent inactivation was still evident, this suggested that alternative mechanisms still may be responsible for controlling these events (Scott et al., 1991, Imredy and Yue, 1994). In 1994, Imredy and Yue proposed a model whereby allosteric changes in channel conformation occurs favoring the closed channel state when calcium binds directly to the channel. After calcium channel structure was determined with the cloned channels, regions of the C-terminus and the calcium binding EF-hand of the α_1 subunit

were then implicated as the mechanism of calcium-dependent inactivation (de Leon et al., 1995; Soldatov et al., 1997, Zhou et al., 1997, Soldatov et al., 1998; Zuhlke et al., 1999). Recently it was suggested that calmodulin acts as the sensing mechanism to promote calcium-dependent inactivation of L-type channels (Peterson et al., 1999; Zuhlke et al., 1999).

Skeletal L-type channels play a role in excitation-contraction (E-C) coupling (Leong and MacLennan, 1998). E-C coupling involves direct interaction of the calcium channel to the ryanodine-sensitive calcium release channel (RyR) of the sarcoplasmic reticulum (Adams and Beam, 1990, Catterall, 1991, Rios and Pizarro, 1991) and the II-III loop of the α_1 subunit (Tanabe et al., 1988, Tanabe et al., 1990a). An action potential within the skeletal muscle transverse tubule initiates L-type channel activation which in turn, activates the RyR channel through protein-protein interactions, causing intracellular calcium concentration to increase and thus muscle contraction. Mice lacking the RyR have normal levels of L-type channels present, but decreased calcium currents suggesting the RyR is required for normal channel function (Nakai et al., 1996, Grabner et al., 1999).

In endocrine cells, L-type channels are involved in the secretion of hormones (Milani et al., 1990). In neurons, L-type channels are localized in cell bodies and proximal dendrites and have been shown to play a role in synaptic input integration, including gene expression (Bean, 1989a, Murphy et al., 1991). More recent evidence suggests L-type channels may play a role in neurotransmitter secretion (Bonci et al., 1998, Protti and Llano, 1998), although the N- and P/Q-type channels have been predominately recognized for this function.

The first calcium channel characterized both biochemically and by molecular techniques was the L-type from rabbit skeletal muscle (Flockerzi et al., 1986, Cooper et al., 1987, Leung et al.,

1987, Takahashi et al., 1987, Tanabe et al., 1987, Campbell et al., 1988, Catterall et al., 1988, Leung et al., 1988, Sharp and Campbell, 1989). Utilizing DHP affinity labeling, the α_1 subunit was isolated (Beam et al., 1992) and expression in dysgenic mice myotubes restored excitation-contraction and provided calcium channel current (Tanabe et al., 1988). These studies showed that the skeletal muscle α_1 subunit could function as a voltage sensor and respond to membrane depolarizations leading to intracellular calcium release and muscle contraction.

Purification of the α_1 subunit from rabbit skeletal muscle T-tubules under mild conditions isolated a number of proteins identifying the L-type calcium channel to be a heteromultimeric complex consisting of five subunits, α_1 (175 kDa), α_2/δ (143 and 27 kDa), β (52 kDa) and γ (32 kDa) (Catterall et al., 1989). Two different sizes of L-type α_1 subunits were purified in skeletal muscle (214 kDa and 193 kDa, De Jongh et al., 1991) and neurons (210-235 kDa and 190-195 kDa, Hell et al., 1993). Subsequently, the rabbit L-type α_1 subunit was isolated from cardiac tissue (Hosey et al., 1989, Norman et al., 1994) and the N-type channel from brain (Witcher et al., 1993b).

The T-type Channels

Low threshold calcium currents were first described in guinea pig inferior olivary nucleus (soma and proximal dendrites) and thalamic neurons (Llinas, 1988). These currents were named T-type for their characteristic "transient" current, small conductance and slow deactivation kinetics. T-type channels have since been found in many cell types including proliferating cells of the cardiovascular and endocrine system (Ertel and Ertel, 1997). T-type calcium channels peak at membrane potentials close to resting, inactivate quickly from 14 to 80 msec, at negative membrane potentials (V_{50}), -110 to –50 mV. The conductance for T-type channels ranges from 7

to 10 pS and in contrast to HVA channels they tend to exhibit equal or greater permeability for Ca^{2+} over Ba^{2+} ions (Bean, 1989, Tsien et al., 1991, Perez-Reyes, 1998, Perez-Reyes et al., 1998).

T-type channels are believed to be involved in neuronal oscillatory activity (Llinas, 1988) and play a role in pacemaker activity by low-threshold calcium spikes and rebound burst firing (Hagiwara et al., 1988, Avery and Johnston, 1996, Ertel and Ertel, 1997). T-type channels have also been associated with smooth muscle contraction (Akaike et al., 1989), and hormone secretion (Cohen et al., 1988; Enyeart et al., 1993). In development, the LVA T-type channel has been demonstrated to be directly involved in fertilization in both invertebrates and mammalian systems (Arnoult et al., 1996; Benoff, 1998).

T-type channel currents are more sensitive to Ni^{2+} than the other calcium channels and are also blocked by relatively high concentrations of octanol, amiloride, carbamazepine and phenytoin (Dolphin, 1995, Perez-Reyes, 1998, Perez-Reyes et al., 1998). Several LVA currents found in mouse thalamic neurons and rat hippocampal CA3 pyramidal neurons exhibit a unique pharmacology (Avery and Johnston, 1996, Ertel and Ertel, 1997).

The P/Q-type Channels

P-type channels were named after cerebellar Purkinje neurons, the location where they were originally characterized (Llinas et al., 1989; Mintz et al., 1992a). Q-type channels were named "Q" because that was the next letter in the alphabet. They were analyzed in cerebellar granule neurons and are designated into a separate calcium channel category and grouped with P-type channels (Mintz et al., 1992a, Mintz et al., 1992b, Zhang et al., 1993, Randall and Tsien, 1995). P- and Q-type channels are both HVA, insensitive to DHPs and ω-conotoxin GVIA (ω-CTX

GVIA) and both are blocked by a 48 amino acid peptide toxin ω-agatoxin IVA from the funnel web spider (ω-Aga IVA).

P-type channels were first shown to be blocked by another funnel web spider toxin FTX (Llinas et al., 1989; Usowicz et al., 1992). P-type currents are blocked by ω-Aga IVA with a Kd of 2 to 10 nM, whereas Q-type channels require higher concentration, Kd of 100 nM (Artalejo et al., 1992, Mintz et al., 1992a, Mintz et al., 1992b, Snutch and Reiner, 1992, Fisher and Bourque, 1995; Pearson et al., 1995). Blocking of P-type current with Aga IVA can be reversed with membrane depolarization (Mintz et al., 1992a). Changes in the degree of blocking are strongly voltage dependent, which suggest conformational binding dependence. To date, no specific blocking compound is known to distinguish the P- and Q-type currents and native currents are determined based on toxin sensitivity differences. P/Q-type channels are blocked irreversibly by a peptide from the marine snail *Conus magus,* ω-conotoxin MVIIC, which blocks N-type channels in a reversible manner (Sather et al., 1993, Zhang et al., 1993, McDonough et al., 1996).

P-type currents have shown little inactivation in Purkinje cells (Llinas et al., 1989; Usowicz et al., 1992) and slow inactivation in other cerebellar neurons (Tottene et al., 1996) while Q-type currents completely inactivate (Pearson et al., 1995). Generally, the P/Q-type channels are described as slow inactivating and have a V_{50} of inactivation ranging between -45 to -5 mV with Ba^{2+}, conductances between 9 and 19 pS, and first activate at greater than –40 mV. Native P/Q-channels are localized along dendrites and presynaptic terminals of neurons (Westenbroek et al., 1992).

The R-type Channels

R-type channels were first characterized in cerebellar granule cells but have since been found in many neuronal cell populations (Randall, 1993a). R-type channels were named after the "residual" calcium current which remained after application of nimodipine, ω-CgTx, ω-Aga IVA and ω-CTx MVIIC in cerebellar granule cells (Zhang et al., 1993). R-type channels have a conductance of 14 pS, activating at greater than –40 mV with a V_{50} inactivation range from –100 to –40 mV. R-type channels inactivate quickly, 20 to 40 msec. In granule neurons, R-type currents were considered HVA and therefore distinguished from T-type channels (Randall and Tsien, 1995). These channels are thought to include multiple channel subtypes and predicted to play a role in neurotransmitter release and somatodendritic excitability (Tottene et al., 1996, Wu et al., 1998, Wu et al., 1999). R-type currents lack sensitivity to all specific blockers and can only presently be distinguished pharmacologically by their increased sensitivity to Ni^{2+} (Zhang et al., 1993).

The N-type Channels

N-type channels, originally believed to be exclusive to the nervous system, have since been found in human and rat endocrine cells as well as rat kidney and liver (Sher et al., 1990, Guthrie, 2001). The original findings led to the naming, "N" for "neither" L- nor T-type current. Using Ba^{2+} as the charge carrier, N-type channel conductances range from 10 to 22 pS. The channels first activate around –30 mV and exhibit an inactivation range (V_{50}) of –120 to –30 mV. The inactivation rate of N-type channels is generally greater than 500 msec. N-type channels undergo time-dependent inactivation, slower than T-type currents but faster than L-type currents, although variability exists between native populations (Nowycky et al., 1985, Bean, 1989, Biagi and Enyeart, 1991, Plummer and Hess, 1991, Jones and Elmslie, 1992). In addition, N-type channels undergo voltage-dependent inactivation, with more negative membrane potentials causing more robust inactivation compared to L-type channels (Nowycky et al., 1985, Fox

et al, 1987a, Fox et al., 1987b, McCarthy and TanPiengco, 1992).

N-type channel currents are sensitive to ω-conotoxin GVIA (ω-CgTx), a toxin from the piscivorous marine mollusk, *Conus geographus* (Plummer et al., 1989). ω-CgTx exhibits a high affinity and irreversible block of N-type channels at low concentrations, 100 nM (Feldman et al., 1987; Kasai et al., 1987; McCleskey et al., 1987, Aosaki and Kasai, 1989, Plummer et al., 1989). Biochemically, the N-type calcium channel was initially purified from rabbit brain using antibodies generated against the skeletal muscle L-type channel in combination with ω-CgTx GVIA binding (Sakamoto and Campbell, 1991, Witcher et al., 1993). The α_1 subunit was purified as 2 different sizes from rabbit brain (235 and 210 kDa, Westenbroek et al., 1992), along with a β subunit (57 kDa), and $\alpha_2\delta$ subunits (160 kDa) (Witcher et al., 1993b).

The exact roles of N-type channels in the nervous system are not clear. The subcellular localization at presynaptic central synapses suggests N-type channels contribute to neurotransmitter release and additional functions most likely exist since N-type channels are also localized to other structures such as dendrites. Application of ω-CgTx to study the effects of neurotransmitter release within the central and peripheral nervous system have shown N-type channels do play a role (Kerr and Yoshikami, 1984, Dooley et al., 1988; Lundy and Frew, 1988, Dutar et al., 1989; Herdon and Nahorski, 1989, Takemura et al., 1989, Wessler et al., 1990, Horne and Kemp, 1991; Burke et al., 1993, Potier et al., 1993).

Interestingly, application of ω-CgTx to different neurons in different species has shown variability in neurotransmission inhibition and has only demonstrated partial block suggesting other calcium channels may be involved. P/Q-type channels have shown similar blocking of

neurotransmission first described in cerebellar and spinal cord preparations with the use of ω-Aga IVA (Mintz et al., 1992b, Takahashi and Momiyama, 1993). Both P/Q- and N-type channels have been localized at the neuromuscular junction (Robitaille et al., 1990, Sugiura et al., 1995) and may each contribute to this process.

Although the study of native calcium channels has given information on expression patterns, channel pharmacology and channel kinetics, there are limitations to the information given. Biophysical, biochemical and molecular biological studies need to be applied to address questions such as what components make up a calcium channel, how it spans the membrane, what the structure looks like when the channel is open versus closed, where channel blockers bind and what modulates channel function in neurons. It appears that more than one type of calcium channel may be involved in neurotransmission and although it is not clear why this is the case, analysis of cloned channels will help resolve this issue and provide further understanding of this process.

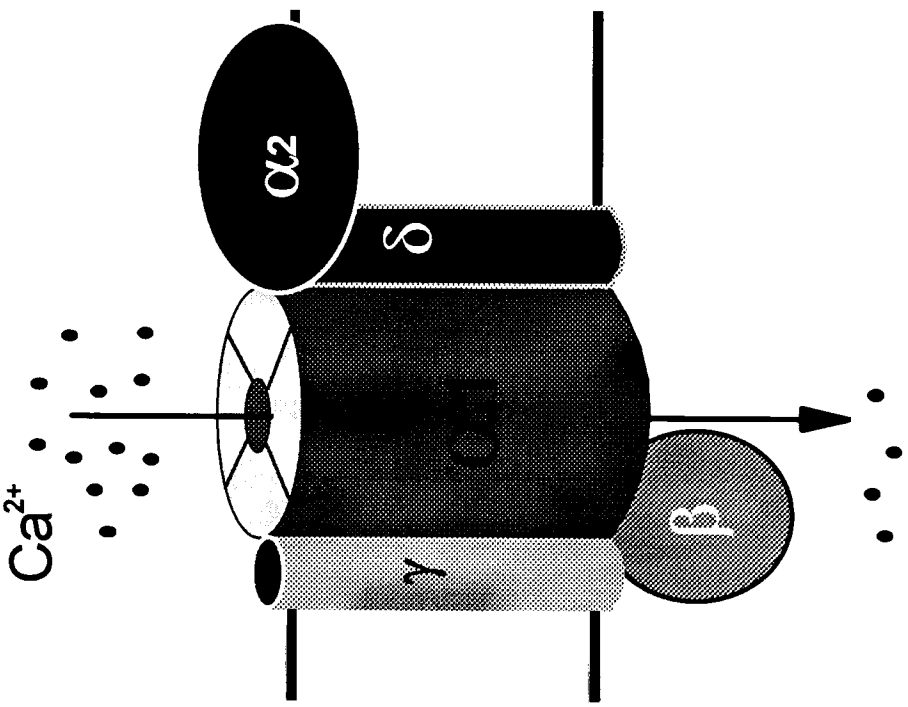
Cloned Calcium Channels

In order to carry out structure-function studies with ion channels, cloning of the channel subunits began. Knowing the amino acid sequence of the proteins that constitute an ion channel has several advantages. For example models of structure can be examined and regions of similarity within protein families gives information on how that channel functions. Genetic engineering enables chimera studies, site-directed mutagenesis and natural mutations causing disease between different channels can be studied in order to determine regions of importance for specific channel properties.

Figure 3 schematically illustrates the proposed organization of the skeletal muscle L-type

Figure 3. Calcium Channel Subunits.

A model of voltage-gated calcium channel subunit composition was proposed from the initial purification of the L-type channel from skeletal muscle (Dolphin, 1995). Further cloning and sequencing from heart and brain revealed the main pore forming transmembrane α_1 subunit associated with the auxiliary subunits; the β subunit, $\alpha_2\delta$ subunit and γ subunit to form a heteromultimeric complex localized to the cell membrane (Campbell et al., 1988, Catterall, 2000).


α2
δ
β
γ
α1
Ca2+

calcium channel complex (molecular weight ~ 390 kDa, Dolphin, 1995). After the identification of the first channel, the skeletal L-type with the α_1, $\alpha_2\delta$, β and γ subunits, isolation of the subunits began using a combination of techniques based on pharmacological sensitivity and specific antibodies (Tanabe et al., 1987, Ellis et al., 1988, Ruth et al., 1989, Jay et al., 1991). Recordings from the central nervous system (Mintz et al., 1992a, Mintz et al., 1992b, Usowicz et al., 1992, Ellinor et al., 1993) led to the official naming of calcium channels in 1990 resolved by Snutch et al., although new nomenclature using numbers versus letters has been introduced (Ertel et al., 2000).

To date, there have been ten classes of α_1 subunit genes identified (α_{1A} - α_{1I} and α_{1S}) (Snutch et al., 1990, Singer et al., 1991, Wei et al., 1991, Perez-Reyes et al., 1992, Soong et al., 1993, Lee et al., 1999a, Talley et al., 1999) and multiple isoforms of each α_1 subtype exists (Hui et al., 1991, Snutch et al., 1991, Diebold et al., 1992, Westenbroek et al., 1992, Williams et al., 1992, Catterall, 1993, Soong et al., 1993, Welling et al., 1993, Dubel, 1994). The gene locations and some diseases associated with these subunits have been identified (**Table 2**). The α_{1A} - α_{1I} subunits are all expressed in the nervous system, while α_{1S} subunits are specific to skeletal muscle (Ellis et al., 1988, Morton and Froehner, 1989, Snutch et al., 1990, Soong et al., 1993). Whole cell and single channel recordings of cloned channels expressed in *Xenopus laevis* oocytes and human embryonic kidney (HEK) cells have led to the correlation between the functional and pharmacological properties of the various cloned channels and native calcium currents. These model systems do not express calcium channels endogenously.

Table 3 summarizes the characteristics of the cloned calcium channels and **Figure 4** indicates the evolutionary relationship among the α_1 subunits. The N-type α_{1B} subunit is closely related to

Table 2. Voltage-gated Calcium Channel α_1 Subunits.

Advances in molecular biology have led to identifying ten different α_1 subunit genes which encode 5 types of voltage-gated calcium channels (van den Maagdenberg, 2001). The gene location and some associated diseases are now known. The novel name for calcium channels is given (Ertel et al., 2000), although this thesis uses the traditional nomenclature (Snutch et al., 1990). 1. hypokalemic periodic paralysis. 2. malignant hyperthermia susceptibility. 3. X-lined congenital stationary night blindness. 4. familial hemiplegic migraine. 5. episodic ataxia type 2. 6. spinocerebellar ataxia type 6.

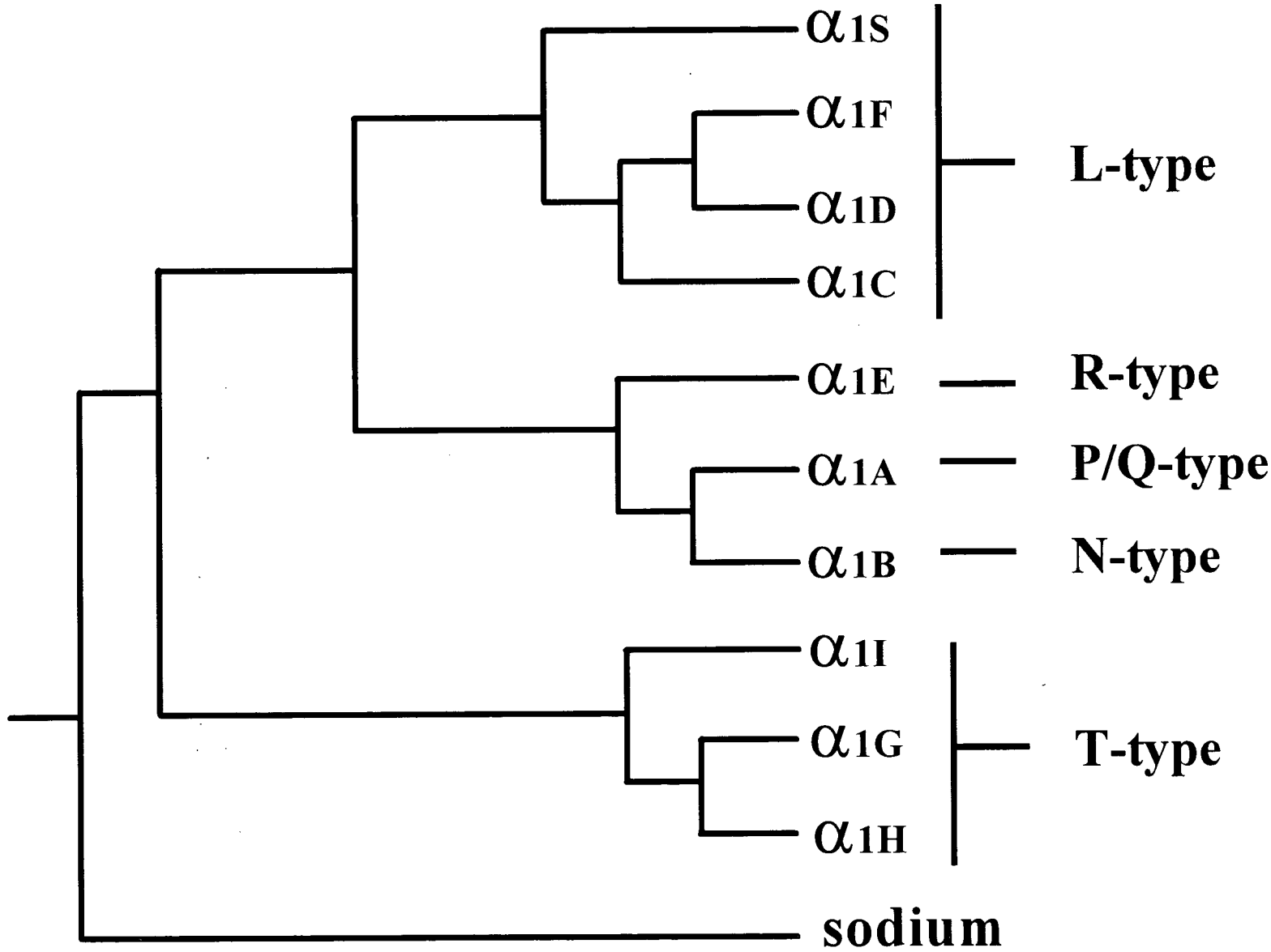
Channel Type	α_1 subunit	Novel Name	Gene	Chromosome	Associated Disease
L	α_{1S}	$Ca_v1.1$	CACNA1S	1q31-q32	HypoPP[1], MHS5[2]
L	α_{1C}	$Ca_v1.2$	CACNA1C	12p13.3	-
L	α_{1D}	$Ca_v1.3$	CACNA1D	3p14.3	-
L	α_{1F}	$Ca_v1.4$	CACNA1F	Xp11.23	CSNB2[3]
P/Q	α_{1A}	$Ca_v2.1$	CACNA1A	19p13.1	FHM[4], EA-2[5], SCA6[6]
N	α_{1B}	$Ca_v2.2$	CACNA1B	9q34	-
R	α_{1E}	$Ca_v2.3$	CACNA1E	1q25-q31	-
T	α_{1G}	$Ca_v3.1$	CACNA1G	17q22	-
T	α_{1H}	$Ca_v3.2$	CACNA1H	16p13.3	-
T	α_{1I}	$Ca_v3.3$	CACNA1I	22q12.3-q13.2	-

Table 3. Properties of Cloned Calcium Channels.

Ten α_1 subunit genes have been identified (α_{1A} - α_{1I} and α_{1S}). Cloned calcium channels expressed in *Xenopus oocytes* or Human Embryonic Kidney (HEK) cells have been categorized based on electrophysiology and pharmacological properties. Values described in Table 3 were approximated using single channel recordings. Results vary based on species, cells, subunit combinations and recording conditions (Schneider et al., 1994, Catterall, 2000). Generally high voltage activated (HVA) use 20 mM Ba^{2+} and low voltage activated (LVA) use 2 mM Ca^{2+} as the charge carrier. DHP, dihydropyridine. ω-CgTx MVIIC, ω-conotoxin from marine snail *Conus magus*. ω-CgTx-GVIA, ω-conotoxin from marine snail *Conus geographus*. Aga-IVA, agatoxin from the funnel web spider *Agelenopsis aperta*. SFTX, synthetic funnel web spider toxin.

Channel Classification (α_1 Subunit)	Steady-state Inactivation V_{50} (mV)	Single Channel Conductance (pS)	Selective Blockers	Localization	Neuronal Localization	Proposed Function
L (α_{1C}, α_{1D}, α_{1F}, α_{1S})	-20	24	DHP antagonists; nifedepine, diltiazem, verapamil, calciseptein, (-)-(S)-BAYK8644, SDZ(+)-(S)-202791, FPL64176.	α_{1C}; cardiac muscle, smooth muscle, brain and pituitary. α_{1D}; brain, heart pituitary, pancreas, PC12 cells. α_{1F}, retina. α_{1S}; skeletal muscle.	Cell bodies and Dendrites.	Gene expression and synaptic integration.
N (α_{1B})	-50	13-20	ω-CgTx GVIA, ω-CgTx MVIIC	α_{1B}; brain, PC12 cells.	Nerve terminals, dendrites and cell bodies.	Neurotransmitter release.
P/Q (α_{1A})	P: -5, Q: -45	10-18	ω-Aga IVA (P: IC_{50} ~1 nM, Q: IC_{50} ~90 nM), ω-Aga IVB (IC_{50} ~3 nM), ω-conotoxin MVIIC.	α_{1A}; brain, heart and pituitary.	Nerve terminals, dendrites and cell bodies.	Neurotransmitter release and gene expression.
R (α_{1E})	-15	14	SNX482, Ni^{2+}	α_{1E}; brain (doe-1 in electric eel).	Cell bodies and dendrites.	Synaptic integration and repetitive firing.
T (α_{1G}, α_{1H}, α_{1I})	-70	8	Mibefradil, Ni^{2+}, flunarizine, furtoxin, SB209712, octanol, amiloride, pinozide and ethosuximide.	α_{1G}; brain and cardiac tissue. α_{1H}; kidney, cardiac and brain. α_{1I}; brain.		Cardiac pacemaker activity, burst firing, hormone secretion, fertilization.

Figure 4. Sequence Homology of Mammalian Voltage-gated Calcium Channel α_1 Subunits. Ten distinct mammalian α_1 subunits and the voltage-gated sodium channel were aligned (domain III and IV, including intracellular loop) and compared using protein parsimony analysis (Felsenstein 1993). Three major groupings were observed; the DHP-sensitive L-type VGCC (α_{1S}, α_{1F}, α_{1D}, α_{1C}) the DHP-insensitive HVA VGCC (α_{1E}, α_{1A}, α_{1B}) and the LVA VGCC (α_{1I}, α_{1G}, α_{1H}). Genebank Accession numbers: α_{1S} - NP_000060; α_{1F} - NP_005174; α_{1D} - NP_000711; α_{1C} - NP_000710; α_{1E} - A37490; α_{1A} - NP_037050; α_{1B} - A45386; α_{1I} - Q9Z0Y8; α_{1G} - O54898; α_{1H} - O95180; sodium - AAA79965 (Stea, 2001).


α1S
α1F
α1D
α1C
L-type
α1E
R-type
α1A
P/Q-type
α1B
N-type
α1I
α1G
α1H
T-type
sodium

the P/Q-type α_{1A} subunit and the R-type α_{1E} subunit. Both the N- and the P/Q-type channels have the strongest evidence suggesting a role in neurotransmission. Dissecting the properties of the clones will help elucidate why more than one calcium channel is responsible for the important process of neurotransmission.

The α_{1S}, α_{1C}, α_{1D} and α_{1F} Subunits

The DHP sensitive α_{1S}, α_{1C} and α_{1D} clones are classified as L-type channels (Perez-Reyes et al., 1989, Williams et al., 1992, Tomlinson et al., 1993). Although the α_{1F} clone has not yet been functionally expressed, based on sequence homology with the α_{1C} and α_{1D} clones, α_{1F} has been grouped with the L-type calcium channels (Strom et al., 1998). The α_{1C} and α_{1D} amino acid sequence share greater than 70 % identity between each other and 66 and 71 % identity with the α_{1S} subunit, respectively. While both L-type channels, the α_{1C} and α_{1D} channels exhibit distinct distribution and pharmacological properties (Snutch and Reiner, 1992).

The α_{1C} was first described in mammalian cardiac tissue (Mikami et al., 1989, Tanabe et al., 1990), but has also been found elsewhere, including rabbit smooth muscle (Biel et al., 1990), rat aorta (Koch et al., 1990), rat brain (Snutch et al., 1991) and mouse brain (Ma et al., 1992). Alternative splicing of the α_{1C} gene has been identified in rabbit lung, rat aorta and rat brain (Biel et al., 1990, Koch et al., 1990, Snutch et al., 1991). The α_{1D} was abundant in rat brain (Hui et al., 1991) and clones have been isolated from human neuroblastoma (Williams et al., 1992b), hamster neuroendocrine (Yaney et al., 1992) and human pancreatic β cells (Seino et al., 1992). Although both the α_{1C} and α_{1D} clones share block by DHP antagonists, the α_{1D} was reversibly inhibited by ω-CTX GVIA, although inhibition was not to the same degree as observed for N-type channels (Williams et al., 1992b).

The rabbit α_{1C} mRNA has been shown to be expressed in heart, brain and stomach by Northern blot analyses (Mikami et al., 1989). The rat aorta α_{1C} mRNA was also found in heart, brain, smooth muscle, uterus, lung, stomach, small intestine and large intestine (Koch et al., 1990). The rat brain α_{1C} was found expressed in the olfactory bulb, cerebellum, striatum, hypothalamus/thalamus, hippocampus, cortex, pons/medulla and spinal cord. Relatively higher detection of α_{1C} was observed in the spinal cord and trigeminal nerve. Both splice forms of α_{1C} were detected in heart, adrenal gland, pituitary, liver, kidney, testes and spleen (Snutch et al., 1991). Mouse α_{1C} was found in heart, brain and spinal cord with no mRNA detected in liver or skeletal muscle (Ma et al., 1992).

The cellular localization of L-type channels has been described by immunohistochemistry using an antibody generated against the rabbit L-type $\alpha_2\delta$ subunit. Clustering of channels was predominant at the base of proximal dendrites, although cell bodies were also stained on hippocampal pyramidal cells, dentate granule cells, pyramidal neurons of the cortex and cerebellar Purkinje and granule cells (Westenbroek et al., 1990). These results were confirmed using a specific α_{1C} antibody, which showed punctate staining on cell bodies as well as proximal dendrites on many central neurons (Hell et al., 1993a).

Northern blot analyses of the human α_{1D} subunit showed mRNA expression predominantly in rat brain and pancreas, but not in skeletal muscle, heart, kidney, spleen, liver, jejunum and colon of human or monkey (Seino et al., 1992). The hamster α_{1D} mRNA levels were detected in pancreas, brain, heart and skeletal muscle (Yaney et al., 1992). RT-PCR showed RNA expression in cortex, cerebellum, hypothalamus and brain stem (Yaney et al., 1992). *In situ* hybridization showed α_{1D} expression in rat brain, olfactory bulb, cerebral cortex, hippocampal CA fields,

dentate gyrus, cerebellar Purkinje cells, suprachiasmatic and supraoptic nucleuses, pituitary and pineal glands (Chin et al., 1992). The cellular localization of rat α_{1D} was similar to that of α_{1C}, high expression on cell bodies and proximal dendrites, although the punctate pattern observed with α_{1C} was not present (Hell et al., 1993a).

The α_{1F} subunit shares 55 to 70 % identity at the amino acid level to the L-type channel subunits with greatest similarity to the α_{1D} subunit and less than 35 % identity to the N- or P/Q-type channels. This clone was isolated first in humans in association with congenital stationary night blindness (Bech-Hansen et al., 1998, Strom et al., 1998). The 219 kDa retina specific protein is thought to be involved in neurotransmission in photoreceptors. The α_{1F} subunit has been found expressed in photoreceptors, horizontal cells, bipolar cells and amacrine cells by in situ hybridization studies (Bech-Hansen et al., 1998, Strom et al., 1998).

Knockout mice missing the α_{1C} subunit die at 14.5 days postcoitum (Seisenberger et al., 2000). A surprising finding in this study was an unidentified L-type current existed in embryonic cardiomyocytes which lacked the α_{1C} subunit. Overall, the study showed the α_{1C} subunit is required for embryo development and the other HVA channels (α_{1A}, α_{1S}, α_{1D}, α_{1F} and α_{1E}) are not (Beam et al., 1986; Bech-Hansen et al., 1998; Jun et al., 1999; Platzer et al., 2000; Saegusa et al., 2000; Seisenberger et al., 2000; Strom et al., 1998).

Knockout mice lacking the α_{1D} subunit were viable, although deaf due to loss of L-type currents in the auditory system, particularly the cochlear inner and outer hair cells (Platzer et al., 2000). Changes in activation kinetics were also apparent. The L-type currents generated from the α_{1D} subunit expression showed low activation thresholds and were slowly inactivated. This is

important in auditory and cardiac pacemaker activity (Platzer et al., 2000).

Human incomplete X-linked congenital stationary night blindness (xICSNB) is a heterogeneous disorder which does not progress. Symptoms include impairment of night vision and reduced day vision. The cause of the disorder has been mapped to mutations of the α_{1F} gene and includes missense mutations, deletions and frameshifts. Vision was believed to be affected as a result of synaptic transmission from photoreceptor cells to second order neurons involving retinal L-type calcium channels.

Hypokalemic periodic paralysis (HypPP) symptoms include weakness of limbs and trunk, without myotonia and a reduction in K^+ levels. HypPP is a dominantly inherited disorder due to missense mutations in the S4 domain of the α_{1S} subunit (R528H in domain II, R1239H/G in domain IV). Malignant hyperthermia (MH) is a dominant muscle disorder, which causes symptoms such as skeletal muscle rigidity, accelerated metabolism, rapid rise in temperature and tachycardia. To date, any of 17 missense mutations in the ryanodine receptor 1 (RyR1) are believed to be responsible for the disease. One case of MH has been demonstrated to be a missense mutation in the α_{1S} gene (R1086H). Central core disease (CCD) shows similar symptoms as MH and also has non-progressive myopathy leading to hypotonia and muscle weakness. Some of the missense mutations found in MH have also demonstrated the onset of CCD.

Cardiovascular diseases are currently being treated with L-type calcium channel blockers which not only block cardiac calcium channels, but also block channels within the midbrain dopaminergic cells (Herman, 1999). Non-insulin-dependent diabetes mellitus (NIDDM) has been well studied in an animal model, Zucker diabetic fatty rats. It appears that the decrease in glucose

induced insulin release is due to a decreased expression of L-type calcium channels (α_{1C} and α_{1D} subunits). These findings may lead to a new approach to treating NIDDM by increasing calcium channel expression (Roe et al., 1996).

Many illnesses are treated with calcium channel blockers. Primary Raynaud's phenomenon includes symptoms of vasospasm of peripheral and visceral organs and is treated with the calcium channel antagonists nifedipine and diltiazem (Belch and Ho, 1996). Withdrawal syndrome of neuroleptics (haloperidol and pimozide) has been treated with nifedipine in rats (Antkiewicz-Michaluk et al., 1995). In studies of drug therapy for acute mania and schizophrenia, an L-type calcium channel blocker (verapamil) has been successful in reducing the symptoms of mania but elevates the symptoms of schizophrenia (Ghosh and Greenberg, 1995).

Currently studies are underway with L-type channel blockers and the treatment of bipolar illness (Bading et al., 1993). Calcium channel antagonists have also been used to study learning and memory and it has been demonstrated that nifedipine leads to an increase in learning and memory activity in schizophrenic patients with tardive dyskinesia (Ginty, 1997). A rat animal model of depression suggests improvement in immobility when rats are treated with verapamil and nicardipine (Davenport and Kater, 1992).

L-type channels are important in muscle contraction, gene expression and synaptic integration. The knockout models suggest a role in development, auditory and cardiac pacemaker activity. Diseases associated with L-type channels also support their predicted function in the nervous system.

The α_{1A} Subunit

The α_{1A} subunit encodes a calcium channel expressed in cardiac and endocrine cells, and shares properties with both P- and Q-type currents (Soong et al., 1993). P-type channels were originally characterized in Purkinje neurons of the cerebellum and found to be blocked by FTX (Llinas et al., 1989; Usowicz et al., 1992) and ω-Aga IVA (Mintz et al., 1992b, Fisher and Bourque, 1995; Pearson et al., 1995). P-type-like currents have also been described with variable properties and are called Q-type (Zhang et al., 1993). Q-type channels differ from P-type channels in their reduced sensitivity to ω-Aga IVA, increased sensitivity to ω-CTx MVIIC and longer inactivation time. Expression of the α_{1A} subunit in oocytes and HEK cells demonstrate channel kinetics and pharmacology which differs from both the native P- and Q-type channels and discrepancies may be due to splice variations of the α_{1A} gene, auxiliary subunit combinations, postranslational processing or protein-protein interactions that are not present under these conditions in these cell types.

Northern blots of rat brain α_{1A} mRNA show two transcripts of 8.3 and 8.8 kb for the rbA-I clone found in cerebellum, spinal cord, pons/medulla, hypothalamus/thalamus and olfactory bulb (Starr et al., 1991). Within the striatum, hippocampus and cortex, the 8.8 kb mRNA is predominant. RT-PCR also showed α_{1A} RNA in rat heart and pituitary with no expression in liver, spleen and kidney. Northern blots of rabbit α_{1A} showed one transcript of 9.4 kb, although two cDNA isoforms have been identified (BI-1 and BI-2, Mori et al., 1991). Cellular localization of the rat α_{1A} subunit has been found in cell bodies of cerebellar Purkinje cells and granule cells (Stea et al., 1994). This was previously shown by immunohistochemistry showing strong labeling of Purkinje dendrites and cell bodies throughout the CNS, including periglomerular cells of the olfactory bulb (Hillman et al., 1991, Llinas et al., 1992).

Knockout mice lacking the α_{1A} subunit showed complete loss of P/Q-type currents and showed increases in L- and N-type currents in Purkinje cell bodies and reduced R-type current in cerebellar granule cell bodies (Jun et al., 1999). Hippocampal slices still undergo neurotransmission with increased presence of N- and R-type channels, supporting the premise that P/Q-, N- and R-type channels all contribute to neurotransmission. Knockout mice underwent premature death with increasing ataxia (Jun et al., 1999; Rhyu et al., 1999) which was not seen in the *tottering* and *leaner* mice, which have disrupted α_{1A} subunit function (Dove et al., 1998; Lorenzon et al., 1998; Wakamori et al., 1998)

Mutations in the α_{1A} subunit cause human diseases such as familial hemiplegic migraine (FHM) that are autosomal dominant (Lorenzon and Beam, 2000). Symptoms include headaches with auras and hemiparesis, ataxia and nystagmus, which have been linked to four missense mutations in the α_{1A} gene (R192Q, T666M, V714A and I181L). Episodic ataxia-2 (EA-2) patients experience ataxia, nystagmus, dysarthria, vertigo and sometimes cerebellar atrophy. Mutations in α_{1A} gene include a frameshift, a single base mutation and a mutation in the 5' splice junction, all of which introduce premature stop codons. Autosomal dominant spinocerebellar ataxia 6 (SCA6) patients experience ataxia, nystagmus, dysarthria and neuronal loss in the cerebellum, dentate and olivary nuclei. This disease is associated with a CAG repeat expansion in the carboxyl terminus of the α_{1A} gene. Episodic and progressive ataxia appear similar to SCA6 and EA-2 but differ in that there is a more prominent cerebellar atrophy and insensitivity to acetazolamide (a treatment used for EA-2 patients). A missense mutation (G293R) in domain I (S5-S6 linker) of the α_{1A} gene is believed to be the cause.

In mice, mutations in the α_{1A} gene correspond to the phenotypes *tottering* and *leaner*

(recessively inherited), which include symptoms of neurological aberrations such as cerebellar function and cerebellar atrophy (Fletcher et al., 1996, Doyle et al., 1997). There are five known *tottering* alleles and the original mutated allele (tg) shows a phenotype of epilepsy, ataxia and episodic dyskinesia. A single nucleotide change (P601L) in domain II (S5-S6 linker) in the α_{1A} gene is believed to be responsible for the phenotype. Another mutant allele (tg^{la}) is phenotypically displayed in leaner mice, which have severe ataxia with no dyskinesia (Herrup and Wilczynski, 1982, Doyle et al., 1997). Loss of cerebellar Purkinje and granule cells are characteristic of leaner mice. The mutation in the α_{1A} gene is a nucleotide substitution in a splice donor sequence changing the isoform to a larger or truncated version, both having altered sequences. *Leaner* mice have decreased whole cell P-type currents in cerebellar Purkinje, shown to be a result of kinetic alterations versus decrease in number of functional channels present (Dove et al., 1998, Lorenzon et al., 1998).

The α_{1E} Subunit

Clones encoding the α_{1E} channel (250 kDa) represent a novel class of calcium channels and have been isolated from rat, mouse, rabbit and human (Soong et al., 1993, Wakamori et al., 1994, Williams et al., 1994, Bezprozvanny et al., 1995). The α_{1E} channel exhibited properties of both HVA and LVA calcium channels (Soong et al., 1993, Williams et al., 1994). These channels are similar to T-type in that they are inactivated near the resting potential and they have equal permeability to Ca^{2+}, Ba^{2+} and Sr^{2+} (Shuba et al., 1991, Bourinet et al., 1996). Interestingly, these channels also show similarity to the HVA currents described as R-type channels in granule neurons (Randall, 1993b). In granule cells, two populations of currents were found with voltage activations of -40 mV and -25 mV (Tottene et al., 1996). The steady state inactivation range (V_{50}) was -22 mV and -4 mV, respectively, which suggest these currents are LVA. Both currents

showed high sensitivity to Cd^{2+} block which suggest they are HVA. Thus, multiple biophysical properties are characteristic of R-type channels and different R-type populations can exist within the same cell.

The pharmacology of this class of calcium channel demonstrated variability, species-specific blockage occurs with both amiloride and ω-Aga IVA (Soong et al., 1993, Schneider et al., 1994). These channels have also been shown to be blocked by Ni^{2+} and partial, reversible block by ω-CTX GVIA (Mori et al., 1991). Recently, a toxin isolated from the venom of the tarantula *Hysterocrates gigas* (SNX482) has demonstrated complete inhibition of HEK cell expressed α_{1E} channels upon application of 200 nM toxin. This compound is not specific to the R-type channel block, partial block was observed with the L-type α_{1C} subunit (Bourinet et al., 2001).

Of the calcium channels identified to date, the α_{1E} channel is the least understood and some controversy exists over characterizing the α_{1E} channel as an R-type calcium channel. Evidence from knock-out mice lacking the α_{1E} channel still showed properties of R-type currents in cultured cerebellar granule neurons (Wilson et al., 2000). These results suggest that although the α_{1E} subunit contributes to R-type currents, it is not responsible for the majority of R-type current and other calcium channel α_1 subunits may play a role. Interestingly, Jun et al. (2000) demonstrated that the R-type current was reduced (80%) in cultured cerebellar granule neurons isolated from α_{1A} channel knock-out mice, although previous antisense experiments do not support this data (Piedras-Renteria and Tsien, 1998).

Northern blots identified two sizes of α_{1E} mRNA in rabbit hippocampus and striatum of 10.5 and 11 kb. Interestingly, only the 10.5 kb mRNA was detected in cerebellum and only the 11 kb

mRNA was detected in the cerebral cortex (Niidome et al., 1992). Rabbit α_{1E} mRNA was also observed at the cerebral cortex, with low levels in the olfactory bulb, midbrain and pons/medulla (Niidome et al., 1992). Human α_{1E} was detected by RT-PCR in kidney, retina, spleen, pancreas and some endocrine cells (Williams et al., 1994). Cellular localization in rat spinal motor neurons was found in the cell bodies and proximal dendrites (Westenbroek et al., 1998). In both human and rat, the α_{1E} channel is localized to neuromuscular junctions, particularly in the muscle fiber membrane (Day et al., 1997).

Knock out mice lacking the α_{1E} subunit showed abnormalities in response to pain (Saegusa et al., 2000) and support the idea that the α_{1E} calcium channel is involved in pain control. Another study showed mice deficient in spatial memory (Kubota et al., 2001). The R-type channel is known to be involved in synaptic integration and repetitive firing with knock out experiments suggesting a role in pain perception.

The α_{1G}, α_{1H} and α_{1I} Subunits

The low-voltage activated T-type channels include the α_{1G}, α_{1H} and α_{1I} subunits. The α_{1G} subunit has been isolated from rat and mouse (Perez-Reyes et al., 1998, Klugbauer et al., 1999, McRory, 1999), the α_{1H} has been isolated from rat and human heart (Cribbs et al., 1998, McRory, 1999), and the α_{1I} subunit has been isolated from rat and human brain (Lee et al., 1999a, McRory, 1999, Mittman et al., 1999). In HEK cells, these LVA channels first activate upon weak depolarizations from negative holding potentials as expected, but have differential permeability to calcium and barium; the α_{1G} and α_{1I} subunits have equal permeability whereas the α_{1H} subunit demonstrates increased permeability to barium versus calcium (Santi, 1999). There are also some differences in IC_{50} for mibefradil and Ni^{2+}, T-type channel blockers (Klugbauer et al., 1999, Santi, 1999).

All three expressed LVA clones have small single channel conductances typical of native T-type channels, $\alpha_{1G} \approx 7.5$ pS, $\alpha_{1H} \approx 5$ pS and the α_{1I} subunit ≈ 11 pS (Lee et al., 1999)

RT-PCR and in-situ hybridizations of the α_{1G} subunit has demonstrated expression within the heart and throughout the brain including cerebellum, hippocampus, thalamus, and olfactory bulb with trace amounts in cerebral cortex and septal nuclei. Some expression was observed in placenta, lung and kidney (Perez-Reyes et al., 1998, McRory, 1999). Some controversy over α_{1H} subunit expression exists in the literature. Originally, this channel was found expressed in cardiac tissue, kidney and liver (Cribbs et al., 1998). Subsequently, expression was found in sensory neurons and to be highly expressed throughout rat brain (Lambert et al., 1998, McRory, 1999). In contrast, RT-PCR has shown the α_{1I} subunit to be specifically expressed in the brain (Lee et al., 1999a, McRory, 1999).

Knock out mice lacking the α_{1G} subunit are viable (Perez-Reyes et al., 1998). The α_{1G} subunit has been suggested to play a role in thalamic relay through a phenomena known as "bursting pacemaker activity" (Kim et al., 2001). Bursting pacemaker activity is when a cell undergoes repetitive action potentials for a small time frame followed by a period of silence. Most cells that are capable of this activity do not require an external stimulus, they are spontaneously active. Bursting pacemaker activity is important in childhood absence epilepsy and the knockout experiments demonstrated a direct role of the α_{1G} subunit in these seizures using the mouse model system. Although T-type channels are known for thier in pacemaker activity in cardiac cells, other roles include hormone secretion and fertilization from non-neuronal cells.

The α_{1B} Subunit

The α_{1B} subunit encodes the N-type channel, characterized by high threshold currents which are irreversibly blocked by ω-CTX GVIA. The α_{1B} channel has been cloned from rat brain (Dubel et al., 1992), human neuroblastoma (Williams et al., 1992a), rabbit brain (Fujita et al., 1993) and the marine ray nervous system (Horne et al., 1993) and found to have highest amino acid similarity to the α_{1A} subunit. The human α_{1B-1} and α_{1B-2}, rabbit BIII and rat rbB-I and rbB-II cDNAs are approximately 90 % identical and encode proteins of 261 to 262 kDa (Stea, 1995).

The rat α_{1B} rbB-1 isoform was first characterized by Dubel *et al.* (1992) using Northern blot techniques and was found to be expressed in neural tissue and cells expressing N-type channels. Using Northern blot and *in situ* hybridization techniques, several groups reported rbB-1 expression in cerebral cortex, hippocampus, forebrain, midbrain, cerebellum and brainstem. Localization was observed within the neuron in dendrites, presynaptic terminals and some cell body staining (Dubel et al., 1992; Westenbroek et al., 1992, Williams et al., 1992a, Fujita et al., 1993; Lin et al., 1997). The BIII mRNA of rabbit was found in the striatum and midbrain (Fujita et al., 1993) and RT-PCR showed human α_{1B-1} and α_{1B-2} RNA to be expressed in the hippocampus, habenula and thalamus (Williams et al., 1992a). The marine ray α_{1B} subunit, doe-4, was found expressed in the electric lobe, with lower expression in the brain (Horne et al., 1993). The rbB-I isoform was found to be localized on dendrites of many neurons as well as axon terminals of mossy fibers of the dentate gyrus granule neurons. Some cell body staining was present in pyramidal cells of the dorsal cortex and Purkinje cells (Westenbroek et al., 1992). The consensus among the various species examined is that expression of the α_{1B} subunits are specific to the nervous system.

The rat brain α_{1B} subunit exists in multiple isoforms (α_{1B-I} and α_{1B-II}), which differ within domain

I, I-II linker and domain III (Williams et al., 1992a, Fujita et al., 1993). Electrophysiological recordings show distinct channel kinetics and current-voltage relations (Stea, 1999). Generally, expression of cloned N-type channels in HEK cells demonstrate currents which activate between -10 and -30 mV, with peak current amplitude at +30 mV. Currents inactivate over time with V_{50} of inactivation at -60mV. As described with native N-type currents, expression of cloned channels are blocked with 1 μM ω-CgTx (Williams et al., 1992a).

Studies in *Xenopus oocytes* of cloned N-type channels showed differences compared to native chnnels in several properties including rates of activation and inactivation. Co-expression of the β subunit further altered these properties (Stea et al., 1993). Although co-expression of the auxiliary $\alpha_2\delta$ and β subunits were required for N-type channel currents in HEK cells (Williams et al., 1992a), expression of the α_{1B} subunit alone in *Xenopus oocytes* resulted in N-type channel currents (Stea et al., 1993). Overall, expression of clones encoding various N-type channel isoforms in oocytes and HEK cells showed characteristic pharmacological and voltage-dependent activation typical of previously described native channels.

Calcium is believed to be a mediator of apoptosis and antagonists have been considered in the treatment of the disease and some evidence supports this approach (Rodnitzky, 1999). In human and animal studies, the use of calcium channel blockers indicates a decrease in neurotoxicity and cells appear to be protected from the apoptosis. Stroke, trauma, epilepsy, pain and diseases such as Huntington's Disease (HD), autoimmune deficiency (AIDS), and amyotrophic lateral sclerosis also consist of cellular apoptosis events mediated by calcium channels and consideration for antagonists in treatment and prevention of neuronal injury is currently being investigated (Kater and Mills, 1991).

Calcium Channel Subunits

The α_1 Subunit

The α_1 subunit functions as the voltage sensor and the Ca^{2+} selective pore, as well as the site of action of many pharmacological agents (Tanabe et al., 1988, Mikami et al., 1989, Perez-Reyes et al., 1989, Biel et al., 1990). The primary structure of the α_1 subunit consists of four homologous hydrophobic domains (domains I, II, III and IV) and each domain has six to eight putative membrane-spanning segments (S1-S6 and SS1-SS2). Other voltage-gated ion channels like sodium and potassium channels, have a similar predicted overall topology (Noda et al., 1984). The transmembrane regions of all known calcium channel α_1 subunits are highly conserved and the majority of sequence diversity exists within the putative intracellular loops and the carboxyl terminus.

The S4 segment in each domain contains positively charged residues in every third or fourth position that are believed to form part of the voltage sensor of the channel. The S4 region of voltage-gated Na^{2+} and K^{+} channels also has been identified as the voltage sensor and involved in channel opening (Stuhmer et al., 1989, Yang and Horn, 1995, Mannuzzu et al., 1996). Two hydrophobic segments between S5 and S6 (SS1-SS2) likely line the channel pore and are involved in ion selectivity (Guy and Conti, 1990). Site directed mutagenesis has been used to identify amino acids responsible for ion selectivity (Yang et al., 1993) and amino acid changes within this region have converted a calcium channel into a Na^{2+} permeable channel (Heinemann et al., 1992).

The β Subunits

To date, four β subunits have been identified (β_1, β_2, β_3 and β_4) which are encoded by four

different genes, all of which are expressed in the brain (Birnbaumer et al., 1994). The β subunits vary in size from 50-85 kDa and splice variants have also been characterized ($\beta_{1(a-d)}$, $\beta_{2(a-d)}$, $\beta_{3(a-dc)}$, $\beta_{4(a-b)}$), (Ellis et al., 1988, Snutch et al., 1990, Mori et al., 1991, Singer et al., 1991, Wei et al., 1991, Hullin et al., 1992, Perez-Reyes et al., 1992, Powers et al., 1992, Castellano et al., 1993, Soong et al., 1993, Castellano and Perez-Reyes, 1994, Morton et al., 1994). Sequence comparison at the amino acid level between the four β subunits reveals high conservation in domains I and II with lower homology within the N-, C-terminus and linker I-II region. One Protein Kinase C (PKC) site and two casein kinase II phosphorylation sites exists in domain I and three PKC sites and two casein kinase II phosphorylation sites are found in domain II in all β subunits (De Waard, 1996). The β subunit is nonglycosylated and hydrophilic and thought to be cytoplasmic (Takahashi et al., 1987).

The expression of α_1 subunits alone in various expression systems has demonstrated the ability to form functional calcium channels (Mikami et al., 1989, Lacerda et al., 1991, Mori et al., 1991, Soong et al., 1993, Stea et al., 1993). The possibility that these systems support channel properties by interacting with endogenous additional channel subunits may be the case and discrepancies between channel classes have been reported. For example, the α_{1B} subunit can exhibit characteristic N-type calcium currents without addition of auxiliary subunits when expressed in oocytes (Stea et al., 1993). In contrast, several studies have shown that the α_{1D} subunit requires the presence of the β subunit when co-expressed in oocytes in order to propagate characteristic L-type channel properties (Williams et al., 1992b, Ihara et al., 1995, Beguin et al., 2001).

The most dramatic effect of co-expressing the β subunit with the α_1 subunit is an increased

current amplitude, which has been observed in all channel types tested to date (Mori et al., 1991, Wei et al., 1991, Williams et al., 1992a, Williams et al., 1992b, Ellinor et al., 1993, Stea, 1993). Expression of the α_{1A} and α_{1B} subunits nicely demonstrates the effects on current amplitude depending on the presence of different β subunits (Stea et al., 1994). Co-expression of the α_{1A} and the β_{1b} resulted in an 18-fold increase in current amplitude. Similarly, co-expression of the α_{1B} or α_{1E} with the β_{1b} resulted in a 4-fold and a 3-fold increase in current amplitude when compared to α_{1B} subunit expression alone in *Xenopus laevis* oocytes (Stea et al., 1993, De Waard et al., 1994, Wakamori et al., 1994). This increase in current amplitude has also been demonstrated with the α_{1A} and α_{1C} subunits when co-expressed with β_1, β_2, β_3 or β_4 subunits, showing a 2- to 19-fold increase (Mori et al., 1991, Wei et al., 1991, Hullin et al., 1992, Perez-Reyes et al., 1992, Castellano et al., 1993, De Waard et al., 1994). These results suggest that the β subunits may act to stimulate or enhance the conductance properties of most α_1 HVA subunits.

Expression of the calcium channel subunits appears to be developmentally regulated and subunit composition in the complex may be altered over time (McEnery et al., 1998, Sher et al., 1998). Some calcium channel complexes have been isolated and show specific α_1 and β subunit combinations. For example, the α_{1S} subunit of rabbit skeletal muscle was co-purified with the β_{1a} subunit and the N-type α_{1B} subunit isolated from human brain was co-purified with the β_3 as well as the β_4 subunit (Pragnell et al., 1991, Witcher et al., 1993a). Not only do channel kinetics change due to an interaction with the β subunit, but channel pharmacology can be altered as well. The α_{1A} subunit has shown differential sensitivity to ω-Aga IVA and a synthetic funnel-web spider toxin (sFTX) with co-expression of different β subunits in HEK cells (Moreno et al., 1997). This finding has led to the hypothesis that the presence of the β subunit causes an allosteric change on the α subunit, structurally altering pharmacological sensitivity.

Structure and functional relationships between calcium channel subunits has been examined through exogenous expression in various systems (Walker and De Waard, 1998). Site-directed mutagenesis and biochemical studies have demonstrated that the β subunit binds to a conserved 18 amino acid sequence (QQ-E- -L-GY- -WI- - -E) within the intracellular loop between domains I and II of the α_1 subunit (Tanabe et al., 1987, Mikami et al., 1989, Mori et al., 1991, Williams et al., 1992a, Williams et al., 1992b, Pragnell et al., 1994). The site on the β subunit which binds to the α_1 subunit is highly conserved between different β isoforms and contains an N-terminal 30 amino acid stretch containing 2 potential PKC phosphorylation sites (De Waard et al., 1994). Although the interaction of the α_1/β subunit has been well characterized, new data suggest other sites of interaction may exist which include the C- and N-terminus of the α_1 subunit (Tsien et al., 1988, Walker et al., 1998, Walker et al., 1999).

Recently, the β_3 subunit was used in the yeast two-hybrid system and found to associate with Kir/Gem, a member of the Ras-related small G-protein family (Beguin et al., 2001). Kir/Gem was found to bind several β subunit isoforms (β_{1-3}) and to calmodulin (CaM). Kir/Gem has an inhibitory effect on both N- and P/Q- type current in BHK cells and inhibits hormone release initiated by L-type channels in PC12 and mouse insulin secreting cell lines (MIN6). It was determined that Kir/Gem was acting through the β subunit to inhibit channel expression by blocking the β subunit function of trafficking the α_1 subunit to the membrane (Beguin et al., 2001).

The β subunit is thought to play a role in channel kinetics (Wei et al., 1991), expression of the α_1 subunit (Perez-Reyes et al., 1989) and assisting in α_1 subunit in transport and/or insertion into the membrane (Chien et al., 1995). Mice lacking the β_1 subunit associated with the skeletal muscle

L-type calcium channel die from asphyxia at birth (Gregg et al., 1996). The phenotype is similar to mice knockouts of the α_S subunit (*muscular dysgenic*, Powell, 1990) and the RyR-1 (*skrr*, Takeshima et al., 1994) in that E-C coupling is absent in the fetus. The L-type currents are decreased in these mice and further analysis showed that this can be attributed to decreased expression of the α_1 subunit. These results suggest that the β_1 subunit is required for transport/insertion of the α_{1S} subunit (Gregg et al., 1996; Strube et al., 1996, Conklin et al., 1999).

Knockout mice with no β_3 subunit exhibited no behavioral, morphological or cellular differences when compared to wild type, although channel kinetics were altered in N-, P/Q- and L-type channels in superior cervical ganglion neurons (Namkung et al., 1998). L- and N-type currents were reduced and P/Q-type channels showed different gating properties. Interestingly, the role of the β subunit and G-protein modulation was examined, but no significant differences were reported in neurons lacking the β_3 subunit (Namkung et al., 1998).

Lethargic (lh) mouse disorder is similar to the human and murine α_{1A} diseases but without neuronal degeneration. An insertion of four nucleotides into the splice donor site of the β_4 gene results in two isoforms which lack the binding site for the α_1 subunit. Interestingly, β_4 subunit knockout mice are lethargic and show neurological disorders similar to that of the *tottering* mice as well as low body weight and defects in non-neuronal tissues such as the immune system (Dung, 1976, Burgess et al., 1997). The β_1 subunit is the only β subunit expressed in skeletal muscle (it is also expressed in the brain). Knockout of the β_1 subunit in mice results in neonatal lethality (Gregg et al., 1996). In contrast, knockout of the β_3 subunit in mice shows no significant phenotypic change other than reduced N-type currents (Smith et al., 1999).

The α_2 and δ Subunits

The α_2 and δ subunits are transcribed from the same gene and are covalently attached post-translationally via a disulfide linkage (Morton et al., 1994). The two subunits are cleaved proteolytically *in vivo* forming a 143 kDa α_2 subunit and a 27 kDa δ subunit (De Jongh et al., 1990, Catterall, 1991b, Jay et al., 1991, Catterall, 1993). Five splice variants of the $\alpha_2\delta$ gene have been reported (Kim et al., 1992, Williams et al., 1992b). Two novel α_2/δ subunits have recently been identified, α_2/δ-2 and α_2/δ-3, which share 56 % and 30 % identity at the amino acid level with the original clone α_2/δ-1 (Klugbauer et al., 1999a). Purification of the rabbit skeletal α_{1S} subunit was found associated with the α_2/δ-1a isoform and the rat α_{1B} subunit of N-type channels was isolated with the α_2/δ-1b isoform (De Waard, 1996).

The α_2 and δ subunits play a role in regulating the α_1 subunit functional properties and have also been shown to increase the numbers of channel complexes at the membrane (Brust et al., 1993, Isom et al., 1994). The $\alpha_2\delta$ subunit can also increase current amplitude; the co-expression of the $\alpha_2\delta$ subunit with the α_{1D} and β_2 subunit in oocytes causes an increase of 1.5-fold, while a 17-fold increase was observed when the $\alpha_2\delta$ subunit was expressed with the α_{1C} and β_{1a} subunit (Singer et al., 1991, Williams et al., 1992b). The $\alpha_2\delta$ has been suggested to play a role in activation and inactivation time course; this has been observed when co-expressed with the α_{1C} subunit of cardiac cells, but not the brain isoform or in HEK cells (Singer et al., 1991, Brust et al., 1993, Tomlinson et al., 1993). A shift in voltage dependence of inactivation has been observed with the co-expression of the α_{1E} with the $\alpha_2\delta$ subunit (Wakamori et al., 1994). In both COS-7 cells and oocytes, the $\alpha_2\delta$ and β1b interact with α_{1G} to increase trafficking or stabilize channels expressed at the plasma membrane (Dolphin et al., 1999b). The co-expression of the γ subunit with the $\alpha_2\delta$ and β subunits changes the kinetics and the voltage-dependence of activation and inactivation of

the α_{1C} subunit (Snutch et al., 1990). The $\alpha_2\delta$ and the γ subunits may also be involved in expression and/or trafficking the α_1 subunit (Mikami et al., 1989).

Malignant hyperthermia susceptibility (MHS) is an autosomal dominant muscle disorder. This disorder has been associated with the ryanodine receptor and more recently, with the α_2/δ subunit located on chromosome 7 (Iles et al., 1994).

The γ Subunit

The γ_1 subunit appears exclusively associated with the L-type channels in smooth and skeletal muscle (Stea, 1995, Jay et al., 1990, Catterall, 1991b). The co-expression of the γ_1 subunit with the cardiac α_{1C} in oocytes results in an increase of current amplitude, different inactivation kinetics and altered voltage-dependence of inactivation (Singer et al., 1991). The cardiac α_{1C} showed further changes in kinetics when the γ and the β_{1a} subunits were expressed in oocytes (Wei et al., 1991). The brain-specific γ_2 subunit was first identified in *stargazer* mice (Letts et al., 1998). More recently, $\gamma_{3\text{-}5}$ subunits have been identified in human (Burgess et al., 2001) and characterized in oocytes for functional impact on the P/Q-type channel. Only minor changes were detected in P/Q-type channel kinetics, but when different the β subunits were co-expressed in combination with the γ subunits, an increase in slow current inactivation resulted (Rousset et al., 2001).

Knock out mice missing the γ_1 subunit are viable and express a different phenotype than the other members of the skeletal L-type channel complex knockouts (α_{1S}, Adams and Beam, 1990; Chaudhari, 1992 and β_1, Gregg et al., 1996). Current traces in skeletal muscle myotubes lacking the γ_1 subunit showed an increase in L-type calcium current amplitude (Freise et al., 2000).

Interestingly, L-type single-channel conductance was not altered, although steady-state inactivation was shifted to the right and time-dependent inactivation was decreased. Thus, the γ_1 subunit appears to alter channel activity by increasing channel sensitivity to voltage resulting in faster inactivation. Modulation of the skeletal muscle L-type channel by the γ_1 subunit could enhance repetitive depolarizations at high frequency and thereby increase contractile force of muscle fibers (Freise et al., 2000). Stargazer (stg) is a recessive mouse disorder and mice experience ataxia, head tossing and absence of seizures. Stg is caused by a disruption in the γ_2 subunit coding region. **Table 4** summarizes the calcium channel knockouts generated to date.

Calcium Channel Modulation

Although native and cloned channel complexes are consistent in their overall pharmacological properties, differences in ion selectivity, permeability and gating have been detected. Voltage dependence, channel run down and inactivation rate are characteristics of calcium channel properties not demonstrated in expressed L- or N-type cloned channels (Flockerzi et al., 1986, De Waard et al., 1994). There was always concern that purification protocols do not isolate the complete complex and other factors may be required to provide the same properties observed in native channels. Searching for novel protein interactions by more sensitive techniques may ultimately lead to identifying further interactions. A more favorable approach to studying ion channels was the reconstitution of purified protein in an in vivo environment supporting modulation of channels by various cellular proteins (De Waard, 1996).

Calcium channels are responsible for many crucial cellular processes such as contraction, secretion, neurotransmission, enzyme regulation and gene expression. These processes are carried out by converting electrical signals at the cell membrane into signal transduction events by calcium influx through voltage-gated calcium channels and thus regulation of calcium

Table 4. Voltage-gated Calcium Channel Knock-out Mice.

Knockout mice were generated by disrupting calcium channel subunit genes, including the α_{1A} (Jun et al., 1999), α_{1B} (Saegusa et al., 2001), α_{1C} (Seisenberger et al., 2000), α_{1D} (Platzer et al., 2000), α_{1E} (Kubota et al., 2001; Saegusa et al., 2000; Wilson et al., 2000), α_{1G} (Kim et al., 2001), α_{1S} (Adams and Beam, 1990; Chaudhari, 1992, Conklin et al., 1999), β_1 (Beurg et al., 1997; Conklin et al., 1999; Gregg et al., 1996; Strube et al., 1996), β_3 (Namkung et al., 1998) and the γ_1 (Freise et al., 2000). Using knockout models, several conclusions for subunit functions were described.

Calcium Channel Subunit	Phenotype	Conclusions
α_{1A}	progressive ataxia and premature death (week 4)	Functions in neurotransmission. Complete loss results in upregulation of other channels.
α_{1B}	partially lethal, 70% survive to adult	Function in neuropathic pain perception.
α_{1C}	die before 14.5 postcoitum	Function in embryonic development.
α_{1D}	viable, deaf	Function in auditory and cardiac pacemaker activity.
α_{1E}	viable	Functions in pain control.
α_{1G}	viable	Function in burst firing and absence seizures.
α_{1S}	perinatal lethal	Functions in E-C coupling.
β_1	perinatal lethal	Functions in E-C coupling and transport/membrane insertion of the α_1 subunit.
β_3	viable	Alters L-, N- and P/Q-type channel kinetics. Increases current amplitude of L- and N-type channels and functions in P/Q-type "willing" state of voltage dependence.
γ_1	viable	Alters L-type inactivation kinetics, increases channel sensitivity to voltage resulting in fast inactivation.

channels contributes to essential cellular functions. Calcium channels do not only undergo intrinsic voltage-dependent modulation through gating mechanisms, but are also regulated by signal transduction pathways. The present understanding is that the L-type current is known to be regulated by protein phosphorylation and the N-, P/Q- and R-type currents are regulated directly by G-proteins. Models of calcium channel modulation are expanding and the molecular mechanisms underlying these processes are currently under investigation.

PKA

Noradrenaline was the first neurotransmitter shown to have an effect on calcium channel activity. Cardiac calcium channels are phosphorylated by Protein Kinase A (PKA) via activation of a β-adrenoceptor resulting in an increase in current (Cachelin et al., 1983, Bean et al., 1984). This process is now understood to involve the GTP-binding protein G_s which serves to activate adenylyl cyclase leading to increased cyclic adenosine monophosphate (cAMP) levels within the cell which in turn activate PKA and phosphorylate the channel. Enhancing calcium current in cardiac tissue results in increased contraction, beat rate and action potential amplitude (Reuter, 1967; Reuter, 1974).

The cardiac α_{1C} subunit does not contain the conserved phosphorylation sites of the skeletal muscle α_1 subunit and purification of the calcium channel β subunit from cardiac myocytes indicated it as the PKA phosphorylation target (Haase et al., 1993). The β subunit is also known to be modulated by PKC and cyclic guanosine 3'-5'-monophosphate (cGMP)-dependent protein kinase (Jahn et al., 1988). The neuronal α_{1C} exists as a small and large isoform and only the large isoform is a substrate for cAMP dependent kinase phosphorylation (Hell et al., 1993b), increasing open probability (Cachelin et al., 1983) and mean open time (Yue et al., 1990).

Voltage-dependent modulation of skeletal muscle L-type channels occurs via PKA and requires a protein kinase A anchoring protein (AKAP, Johnson et al., 1997).

cAMP

Muscle contraction is modulated by the cAMP signaling pathway activated by epinephrine and calcitonin gene-related peptide (Cairns and Dulhunty, 1994; Catterall, 2000; Fleming et al., 1993). Skeletal muscle α_1 and β subunits are substrates for cAMP-dependent protein phosphorylation, resulting in increased number of functional channels in reconstituted phospholipid vesicles (Mundina-Weilenmann et al., 1991) and single-channel recordings reveal increased number and activity of the channel (Flockerzi et al., 1986; Hymel et al., 1988). Cultured skeletal muscle cells show enhanced calcium currents upon repetitive depolarization which is depended upon voltage and cAMP-dependent protein kinase (Sculptoreanu et al., 1993). Ultimately, this regulatory effect enhances calcium channel activity during tetanic stimulation in response to motor nerve or hormone input resulting in efficient muscle contraction.

Neuronal populations of native calcium channels are substrates for cAMP-dependent kinase including L-type channels of hippocampal and cerebellar granule neurons (Dolphin, 1995), isolated P-type currents expressed from rat cerebellum RNA in oocytes (Fournier et al., 1993) and N-type channels purified from rabbit brain (Ahlijanian et al., 1991). Native calcium current populations exhibited an apparent increase in current amplitude in cultured rat sensory neurons with increases in cAMP levels (Dolphin et al., 1989) and cAMP was believed to act on the steady-state inactivation of the channel in cultured rat dorsal root ganglion cells and oocytes expressing P-type channels (Dolphin, 1991, Dolphin, 1992, Fournier, 1993).

CAM Kinase II

Two splice varients of the N-type α_{1B} subunit are known (220 and 250 kDa) and differ in the C-terminus (Westenbroek et al., 1992). Although both isoforms are phosphorylated by cAMP-dependent kinase, cGMP-dependent kinase and PKC, only the larger isoform is a substrate for calcium and calmodulin-dependent protein kinase II (CaM kinase II, Hell et al., 1994). With the differential phosphorylation patterns of N-type channel splice varients, a neuron is capable of multiple levels of regulation.

PKC-dependent Modulation

PKC modulation of calcium channels appears controversial, although the method of activating the PKC pathway may cause discrepancies. Initial work showed that application of phorbol esters results in an increase in calcium channel current in cardiac cells and sympathetic neurons (Lacerda et al., 1988, Lipscombe et al., 1988), but phorbol esters were also shown to inhibit channel activity in chick sensory neurons using a non-PKC pathway (Hockberger et al., 1989). The cardiac L-type channel was the first identified as being phosphorylated by PKC by expressing heart RNA in oocytes (Bourinet et al., 1992, Singer-Lahat et al., 1992). Both N- and L-type channels show enhanced current in frog sympathetic neurons when the PKC pathway was activated (Yang et al., 1993). Alternatively, N-type channel current in chick sensory neurons is inhibited by activation of the PKC pathway (Cox and Dunlap, 1992).

It has been shown that the N-type channels are directly phosphorylated by PKC (Ahlijanian, 1991) and this results in an increase in whole cell currents. Additionally, PKC affects N-type channel function in neurotransmission by modulating the synaptic vesicle machinery (see below) and playing a role in attenuating the G-protein pathway (Haass et al., 1990, Yokoyama et al., 1997, Cooper et al., 2000). Some of the controversy surrounding PKC modulation as well as other kinases maybe due to calcium channel isoform specificity. For example, the rat neuronal

α_{1C} has two isoforms (190 and 210 kDa, Hell et al., 1993a, Hell et al., 1993b) and the 210 kDa isoform is an *in vitro* substrate for PKA phosphorylation while both isoforms are PKC, CAMK II and cGMP-dependent protein kinase substrates (Hell et al., 1993b).

G-Protein-dependent Modulation

N and P/Q-type channels are known to be regulated by both heterotrimeric G-proteins and PKC-dependent phosphorylation (Dunlap and Fischbach, 1981, Dolphin et al., 1986, Bean, 1989b, Mintz and Bean, 1993, Swartz, 1993, Swartz et al., 1993, Hille, 1994, Rhim and Miller, 1994, Stea et al., 1995, Bourinet et al., 1996, Zamponi et al., 1997). Chimeric cDNA studies have shown that residues in the α_{1A} and α_{1B} domain I-II linkers as contributing to crosstalk between G-proteins and PKC modulation (Zamponi et al., 1997). The $G_{\beta/\gamma}$ binds to the α_1 subunit causing a decrease in response to depolarization (Patil et al., 1996, Zamponi and Snutch, 1998a, Zamponi and Snutch, 1998b) and dissociation occurs with a strong depolarizing prepulse which results in facilitation (Bean, 1989b, Kasai, 1991, Elmslie et al., 1992, Patil et al., 1996). The inhibition of calcium channels by G-proteins is rapid, membrane delimited and has a strong voltage dependence.

G-protein-dependent inhibition has been described in native cells, but it wasn't until the cloned channels were studied that the molecular determinants were described. Work done by Bourinet et al. (1996), showed that the α_{1A} or α_{1B} channels in *Xenopus oocytes* undergo whole cell current inhibition when a μ-opioid receptor agonist (DAMGO) is applied. This inhibition was voltage dependent and at more positive potentials the inhibition was relieved. Investigators were able to facilitate the inhibited current by application of a prepulse to 100 mV for 100 msec before stepping the current to depolarized potentials for 400 msec (**Figure 5,** Bourinet et al., 1996). The

term "facilitation" in neuroscience has been used extensively to explain conditioning responses to stimulus, in particular an enhancement of response. In this instance, calcium currents are facilitated by removal of the inhibiting mechanism caused by activation of the G-protein pathway and restoring current flow to normal levels.

It has been determined that the $G_{\beta/\gamma}$ complex is responsible for the inhibition of calcium channels and the target of the direct physical interaction is largely the I-II linker of the α_1 subunit (Zamponi et al., 1997). The N-terminus/domain I and C-terminus regions have also been implicated in interacting with $G_{\beta/\gamma}$ (Zhang et al., 1996, De Waard et al., 1997, Herlitze et al., 1997, Qin et al., 1997). There is also some evidence that the $G_{\alpha o}$ subunit modulates both N- and L-type channels reducing peak current (Campbell et al., 1995), although no direct evidence has been presented to show that $G_{\alpha o}$ subunits interact with the channel complex.

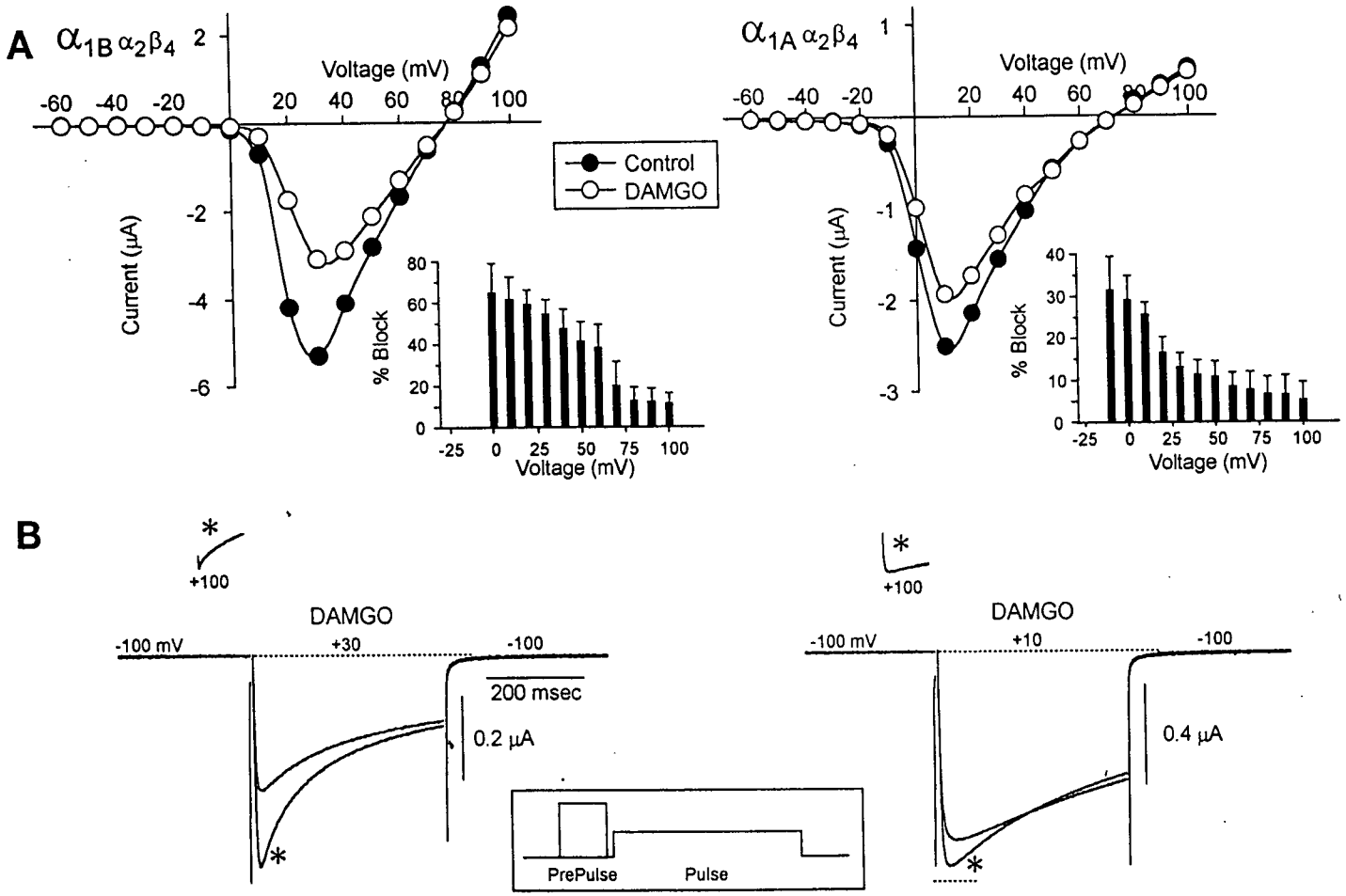
Direct $G_{\beta/\gamma}$ binding has been shown with the α_{1A}, α_{1B} and α_{1E} subunits, which all contain the $G_{\beta/\gamma}$ binding motif (QXXER, De Waard et al., 1997). The $G_{\beta/\gamma}$ pathway can be stimulated by co-expression of the channel with a cloned metabotropic glutamate receptor (mGluR1α) which activates phospholipase C via a G-protein mechanism (Masu et al., 1991, Nakamura et al., 1994). Similarly, activation of somatostatin receptors also activate the G-protein pathway (Zamponi et al., 1997). G-proteins are thought to allosterically modulate both N- and P/Q-type channels, cycling channel states between "willing" and "resistant" states of activation (Herlitze et al., 2001).

The N-type channel undergoes a phenomenon known as "crosstalk" between the calcium channel β subunit and $G_{\beta/\gamma}$ subunit which is known to occur in the I-II linker of the α_1 subunit

Figure 5. Modulation of α_{1A} (P/Q-type) and α_{1B} (N-type) Channels by Opioids.

A. Current-voltage relationships of α_{1A} (right) and α_{1B} (left) expressed in *Xenopus oocytes*. Control situation (without application of μ-opioid receptor agonist DAMGO, closed circles) and application of DAMGO (open circles) are shown demonstrating the inhibition of current when the G-protein pathway is activated. Inset histograms show the inhibition (% block) at various membrane potentials, the more positive the potential, the less current block.

B. Whole cell current traces in *Xenopus oocytes* expressing α_{1A} (left) and α_{1B} (right) with the α_2 and β_4 auxiliary subunits. Currents were recorded with (*) or without a prepulse where a strong 100 msec depolarization to +100 mV was done before stepping the cell to +10 (α_{1A}) or +30 (α_{1B}) for 400 msec. Inset shows the protocol followed. Both channels demonstrate prepulse facilitation with the application of a strong depolarizing prepulse enhancing current flow (Bourinet et al., 1996).


A
$\alpha_{1B}\alpha_2\beta_4$
Voltage (mV)
Current (μA)
Control
DAMGO
% Block
Voltage (mV)
$\alpha_{1A}\alpha_2\beta_4$
B
+100
DAMGO
-100 mV
+30
-100
200 msec
0.2 μA
PrePulse
Pulse
+10
0.4 μA

(Zamponi and Snutch, 1998a, Zamponi and Snutch, 1998b). Binding of the channel β subunit antagonizes the inhibitory effect of $G_{\beta/\gamma}$. The notion of a β subunit crosstalk effect has been discussed concerning the N- and L-type channel inhibition by $G_{\alpha o}$ as well (Campbell et al., 1995).

Recently, a study was done in COS-7 cells whereby co-expression of the β and the α_{1B} subunits showed enhancement of G-protein inhibition, contradicting the results found in HEK cells (Meir et al., 2000). The experiments carried out also suggest the β subunit was required for G-protein-dependent modulation of N-type channels. The $G_{\beta/\gamma}$ complex also undergoes crosstalk with the PKC pathway in the same domain I-II region of the α_{1B} N-type channel (Hamid et al., 1999). In contrast to the G-protein pathway, PKC phosphorylation leads to upregulation of N-type channel activity. Two PKC phosphorylation sites within the I-II linker have been identified, one of which (Thr^{422}) mediates the antagonistic effect of PKC upregulation on G-protein inhibition of calcium channels (Hamid et al., 1999).

Synaptic Vesicle Release Machinery

The α_{1A} and α_{1B} subunits both interact with synaptic vesicle proteins known to be involved in excitation-secretion coupling (E-S coupling) (Tanabe et al., 1990a, Tanabe et al., 1991, Tang et al., 1993, Ellinor et al., 1994, Nakai et al., 1994, Sheng et al., 1994). N-type channels have been shown to contain a "Synprint" site in the domain II-III linker, which binds syntaxin, SNAP-25 and synaptotagmin, proteins involved in vesicle docking and neurotransmitter release (Sheng et al., 1994, Bezprozvanny et al., 1995, Mochida et al., 1996, Rettig et al., 1996, Sheng et al., 1996, Sheng et al., 1997). The interaction is further modulated by serine/threonine kinases; phosphorylation of the Synprint site by PKC or CaM KII inhibits channel interaction with

SNAP-25 and syntaxin (Yokoyama et al., 1997). The P/Q-type channel also interacts with vesicle release proteins. Expression of the α_{1A} rbA isoform binds synaptotagmin and SNAP25 and the BI clone binds all three proteins as well as cysteine string proteins (Rettig et al., 1996, Leveque et al., 1998). In addition to P/Q-type channels binding syntaxin, it has been demonstrated that the influx of Ca^{2+} through the P/Q channels expressed in HEK cells results in an increase in syntaxin transcription (Sutton et al., 1999, appendix).

Clustering Molecules

Various ion channels are known to be clustered along dendrites and at synaptic terminals and proteins likely responsible for clustering are beginning to be identified. The nicotinic acetylcholine receptors are clustered by rapsyn (Bezakova and Bloch, 1998) and glycine and GABA receptors are clustered by gephyrin (Craig et al., 1996; Meyer et al., 1995). The shaker-type K^+ channels and NMDA receptors are clustered by post synaptic density protein of 95 kDa (PSD-95, Niethammer et al., 1996). AMPA receptors are clustered by GRIP (Dong et al., 1997) and metabotropic glutamate receptors are clustered by Homer (Brakeman et al., 1997).

The specific proteins involved in the clustering of calcium channels have not yet been identified but the β subunit has been suggested to play a role (Chien et al., 1995, De Waard, 1996, Brice et al., 1997). More recently, the N-type α_{1B} subunit has been found to associate with a tripartite complex consisting of modular adapter proteins CASK, Mint 1 and Veli via PDZ domain (post synaptic density protein, Drosophila discs large, zonula occludens) of Mint 1 and the carboxyl terminus of the α_{1B} subunit (Maximov et al., 1999). It has also been hypothesized that the carboxyl terminus of the α_{1A} subunit interacts with Homer via a PDZ domain, which may play a role in anchoring/clustering of the P/Q-type channels at presynaptic terminals (data not

published).

Rationale

The hundred billion excitable cells of the nervous system appear to translate electrical signals into action using calcium. Signal transduction and communication processes seem to be modulated by proteins which regulate calcium entry into a neuron, such as voltage-gated calcium channels. The time between the action potential initiation and the release of neurotransmitter is dependent on the opening of calcium channels and the time it takes the intracellular calcium to trigger vesicle release.

N-type calcium channels are involved in neurotransmission. This was first suggested by localization studies, indicating abundant dendrite and axonal terminal localization in DRGs (Nowycky et al., 1985), Purkinje dendrites (Fortier et al., 1991) and at the NMJ (Cohen et al., 1991; Farinas et al., 1993; Robitaille et al., 1990; Torri Tarelli et al., 1991). Single channel recordings on growth cones and synaptic terminals of sympathetic neurons supported this (Lipscombe et al., 1988). Several studies were done showing ω-CgTx binding to N-type channels inhibits neurotransmitter release at the NMJ as well as the central and peripheral nervous system (Dooley et al., 1988; Dutar et al., 1989; Herdon and Nahorski, 1989; Horne and Kemp, 1991; Kerr and Yoshikami, 1984; Lundy and Frew, 1988; Potier et al., 1993; Takemura et al., 1989b; Wessler et al., 1990).

Interestingly, the ability of ω-CgTx block of neurotransmission was variable, depending on the type of synapse and species under study (Horne and Kemp, 1991; Potier et al., 1993; Wessler et al., 1990). This suggested other calcium channels also play a role in this process. P/Q-type channels are the alternate source of calcium influx thought to participate in neurotransmission

and localize within nerve terminals (Westenbroek et al., 1995). P/Q-type channel blocker ω-Aga IVA inhibited neurotransmission at CA3-CA1 hippocampal synapses and therefore the P/Q-type channel was considered to be the calcium channel responsible for vesicle release within the mammalian NMJ (Burke et al., 1993; Dunlap et al., 1995; Luebke et al., 1993; Uchitel et al., 1992; Wheeler et al., 1994; Wu et al., 1999a).

N-type channels were also shown to directly interact with vesicle release machinery syntaxin, SNAP25 and synaptotagmin (Leveque et al., 1994; Leveque et al., 1992; Sheng et al., 1996; Sheng et al., 1994; Sheng et al., 1997; Yokoyama et al., 1997). P/Q-type channels also bind synaptic vesicle machinery such as syntaxin (Rettig et al., 1996) and regulate the expression of syntaxin as well (Sutton et al., 1999). It has been hypothesized that the P/Q-type calcium channel can indirectly modulate the N-type channel by acting through syntaxin. The N-type channel currents are inhibited through the G-protein pathway and in the presence of syntaxin, that inhibition is more pronounced (Bezprozvanny et al., 2000; Degtiar et al., 2000; Jarvis et al., 2000; Jarvis and Zamponi, 2001; Lu et al., 2001).

Both the P/Q-type and N-type channels seem to be intricately involved in the vesicle release process. Both channels are closely related (**Figure 4**) and appear to have similar biophysical properties. A PDZ domain has recently been identified in the N-type α_{1B} (Maximov et al., 1999) and these domains are known to act as the "glue" for stabilizing large complexes (Bezprozvanny and Maximov, 2001). Thus, it was predicted that the N-type α_{1B} subunit may act as a good candidate to use in an attempt to identify novel protein interactions that would help elucidate N-type channel physiological contributions to neurotransmission.

A goal of this thesis was to identify novel proteins that interact with the α_{1B} subunit of the N-

type calcium channel complex. The approach taken was to use the yeast two-hybrid system to detect new interactions. This technique is highly sensitive and may identify interactions that traditional methods have not. The putative cytoplasmic regions of the α_{1B} subunit were used to screen a rat brain library since several protein interactions were already characterized (e.g. binding to the calcium channel β subunit in the domain I-II linker and to syntaxin in the domain II-III linker). Additionally, the cytoplasmic regions are diverse amongst the various cloned calcium channels and may be responsible for unique N-type protein-protein interactions.

Several predictions could be made on what types of proteins may be isolated with the N-type channel. No proteins have been shown to cluster calcium channels and yet the N-type channel has specific punctate staining on dendrites (Coppola et al., 1994; Day et al., 1997; Dubel et al., 1992). It has also been hypothesized that calcium channels are in close proximity to other membrane proteins such as opioid receptors, which indirectly modulate the channel. Proteins that associate calcium channels to their effectors may help with understanding presynaptic organization.

Chapter 2. Materials and Methods

Yeast Two-hybrid System

Background

The yeast two-hybrid system is a yeast-based genetic assay utilized to detect protein-protein interactions *in vivo* (Fields and Song, 1989, Chien et al., 1991, Guarente, 1993, Fields and Sternglanz, 1994, Luban and Goff, 1995, Nishimune et al., 1996). In order to detect proteins that interact, the Gal 4 yeast two-hybrid system uses two domains of the yeast Gal 4 transcriptional activator, the activation domain and the binding domain (Keegan et al., 1986, Fields and Sternglanz, 1994, Bartel and Fields, 1995). Bait PCR products are subcloned into the pAS2-1 plasmid containing the Gal 4 binding domain to form an in frame fusion protein. The "fish" library typically contains fragments of cDNA amplified from RNA, which was subcloned into the pGAD10 plasmid (Bartel, 1993) containing the Gal 4 activation domain. In order to identify protein-protein interactions, both constructs are transfected into yeast and if interaction occurs between the Bait and the library fusion proteins, the two domains of the Gal4 transcriptional activator are united and reporter genes (HIS3 and LacZ) indicate a positive protein-protein interaction.

The pAS2-1 plasmid carries TRP^- and yeast expressing this construct are Trp auxotrophic (require tryptophan in the medium to survive). The pGAD10 plasmid encodes Leu^- and yeast

expressing this construct are Leu auxotrophic (require leucine in the medium to survive). For library screening, yeast cells that have been transformed with both plasmids were plated on synthetic dropout (SD) minimal media to select for specific phenotypes (minimal media = 6.7 g/L Difco yeast nitrogen base without amino acids, 20 g/L Agar, 100 ml of dropout solution containing all but one or more amino acids, 2% dextrose). Yeast cells that grow on SD media lacking Leu and Trp indicate they contain both plasmids. Yeast cells that grow on media lacking histidine (His) are expressing the HIS3 reporter gene which occurs when the Gal4 transcriptional activator is reconstituted. The LacZ reporter gene (which has a different promoter from HIS3) further reduces false positives. LacZ detection was verified by β-galactosidase activity using the colony-lift filter assay described below. The yeast strains used for screening (CG-1945 and Y190) were cycloheximide resistant (Cyh^R) due to the cyh^r2 mutant allele. The CYH2 gene codes for the L29 protein of the yeast ribosome. Cycloheximide prevents cell growth in the presence of the CYH2 gene. The pAS2-1 plasmid carries the CYH^S2 gene, which is dominant and causes yeast strains to become sensitive to cycloheximide and is a mechanism used to isolate pGAD10 constructs (Harper et al., 1993).

Bait Preparation

Seven Bait constructs were generated against the putative cytoplasmic regions of the rat N-type calcium channel α_{1B} subunit and used in screening a yeast two-hybrid rat brain cDNA expression library. Oligonucleotides containing an Eco RI restriction site at the 5' end and a Sal I restriction site at the 3' end were generated to amplify the Bait regions using PCR (polymerase chain reaction, see **Appendix Figure 1 and Figure 2**). The NAPS Unit (Nucleic Acid/Protein Synthesis Unit) at the University of British Columbia (UBC) Biotechnology Lab synthesized all oligonucleotides used in this project (**Appendix, Figure 3**). All Baits were synthesized using a standard PCR protocol with distinct annealing temperatures for the first seven cycles. The Bait

PCR products were subsequently subcloned into the pCRII T-tailed cloning plasmid (Invitrogen) and the DNA sequence determined using the dideoxy nucleotide sequencing method with T7 forward oligonucleotide and SP6 reverse oligonucleotides (**Appendix, Figure 4**). Internal oligonucleotides available in the Snutch Lab for the α_{1B} subunit (SD30 clone, Dubel et al., 1992) were used to confirm the PCR sequence of Bait 2 (oligonucleotides 1C, R27, 1G and R25A). Subsequently, all Bait PCR products were further subcloned into pAS2-1 plasmid and sequence was confirmed with T7 forward and SP6 reverse oligonucleotides.

For use as a positive control in the yeast two-hybrid system, full-length rat syntaxin-1A (SXN; accession AF217191, Bennett et al., 1992) was amplified using RT-PCR (reverse transcription-polymerase chain reaction, **Appendix Figure 5**) and whole rat brain RNA. Forward oligonucleotide HG200A and reverse oligonucleotide HG201 were designed to amplify an 894 base pairs (bp) SXN PCR product by a touch-down protocol ranging from 44°C to 36°C. Eco RI restriction enzyme sites were added to the 5' and 3' ends of the coding region of SXN. After subcloning into the pGem-T Easy plasmid, the SXN sequence was confirmed with T7 forward, SP6 reverse, HG202 and HG203 oligonucleotides. SXN was further subcloned into pAS2-1 and pGAD10 plasmids and the DNA sequence confirmed using forward oligonucleotides for the plasmids.

Library Amplification

A "fish" library obtained from Clontech (catalog number RL4000AB) was generated from whole brain mRNA isolated from 100 outbread Sprague-Dawley male rats (10-12 weeks old). The cDNA was cloned into pGAD10 plasmid at the Eco RI restriction enzyme site with Bgl II restriction sites added by primer extension during amplification. The original titer of the library was 1 X 10^8 colony forming units (cfu)/ml and the titer was confirmed by plating different

dilutions of the library on selective media (1 L of LB = 10 g bacto-tryptone, 5 g bacto-yeast extract, 10 g NaCl, 10 g agar, 50 μg/ml ampicillin). Amplification of the library was carried out by plating 25 μl aliquots of bacterial cells onto 150 mm plates. Bacteria were grown overnight at 37°C and colonies were scraped off and grown in culture in LB broth with ampicillin at 37°C with shaking at 250 rpm (revolutions per minute) for four hours. Library DNA was isolated by Qiagen Giga Plasmid Preparation (Qiagen-tip 10000) and used with all screens.

Protein Expression in Yeast

To confirm Bait expression, 100 ml yeast cultures were grown in SD/Trp$^-$ media overnight. Samples were centrifuged in a microfuge and pellets resuspended in 25 mls dH_2O. Samples (2 ml) were then centrifuged and pellets resuspended in 0.25 M NaOH/1% 2-mercaptoethanol and incubated on ice 10 minutes. Trichloroacetic acid (50% TCA, 160 μl) was added and samples were incubated on ice 10 minutes. Pellets were resuspended in 1 ml acetone, centrifuged and then resuspended in 100 μl of sample buffer (4% SDS, 20% glycerol, 0.12 M Tris (pH 6.8), 0.01% bromophenol blue (BPB), 0.1% dithiothreitol (DTT)). Twenty μl of each sample was loaded onto 8 or 10% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) and Western blotting carried out as described below. Blots were performed with the primary mouse monoclonal Gal 4 DNA-binding domain (BD) antibody was used, which specifically binds amino acids 1 to 147 of the yeast Gal 4 binding domain protein (Clontech).

Generation of Stable Yeast Cell Lines

Two methods of screening were performed; co-transformation (where both the library and the Bait constructs were transformed together into a yeast cell line) and sequential transformation (initially generating stable cell lines containing the Baits and then transforming in the library

constructs). Sequential transformation proved to be the most successful method of screening and resulted in the highest transformation efficiency. Yeast cell lines (Y190/Bait 1, Y190/Bait2, CG-1945/Bait2, Y190/Bait3, Y190/Bait4, Y190/Bait5) were tested to confirm that Bait protein expression was not interfering with the genetic assay by self-activating reporter genes. Growth of yeast cell lines was analyzed on SD selection media; -Leu, -Trp, -Leu/-Trp and -Leu/-Trp/-His. Selections on –Leu/-Trp/-His plates were performed with a titer of 3-amino-1, 2, 4-triazole (3-AT) at 0 mM, 5 mM, 15 mM and 25 mM. The 3-AT acts as a competitive inhibitor of yeast His3 protein and inhibits the low levels of expression of this protein in some leaky reporter strains. β-galactosidase activity was also checked for false activation using a colony filter lift assay (see below). Competent cells were prepared using the lithium acetate (LiAc) method and transformations were carried out as described in Clontech protocols from the MATCHMAKER two-hybrid System 2 kit.

Small Scale Transformation

Y190 yeast cells were plated on YPD plates and approximately 15 colonies were grown overnight in 1 ml YPD media (30°C with shaking at 250 rpm). Fifty mls of YPD media was inoculated and grown for 18 hours at 30°C with shaking at 250 rpm. Three hundred mls of YPD was inoculated to a density of 0.2 to 0.3 at 600 λ and grown for three hours at 30°C with shaking at 250 rpm. Cells were centrifuged and the pellet was resuspended in 50 mls water, centrifuged again and the pellet resuspended in 1 X TE (10 mM Tris-Cl (pH 7.4), 1 mM ethylenediaminetetra-acetate (EDTA, pH 8.0))/LiAc. Bait DNA (0.1 μg) was mixed with library DNA (0.2 μg), salmon sperm DNA (0.1mg), competent cells (100 μl) and polyethylene glycol (PEG)/LiAc (0.6 ml). The solution was vortexed and incubated at 30°C for 30 minutes with shaking at 230 rpm. Dimethyl sulfoxide (DMSO) was added (70 μl) and cells were heat shocked

at 42°C for 15 minutes. Samples were then cooled on ice, centrifuged and the pellet resuspended in TE (0.5 mls). Samples (100 µl) were spread on plates containing selective growth medium.

Large Scale Transformations

Large scale transformations were carried out for library screening. Competent cells were prepared for a co-transformation or alternatively a stable cell line was prepared for sequential transformation as described above. For co-transformation, a typical screen included 100 µg Bait DNA, 50 µg library DNA, 2 mg salmon sperm DNA, and 1 ml competent cells in 6 mls PEG/LiAc solution. Samples were grown for 30 minutes at 30°C with shaking at 230 rpm. Seven hundred µl DMSO was added and samples were heat shocked at 42°C for 15 minutes. Cells were placed on ice five minutes, centrifuged and the pellet resuspended in 10 mls of TE. Three hundred µl aliquots were plated onto 150 mm plates under selection (-Leu/-Trp/-His/25 mM 3-AT) and plates were incubated at 30°C for seven days.

β-galactosidase Assay

A colony-lift filter assay was used to detect activity of the LacZ reporter gene which is expressed if the Gal 4 transcriptional activator was restored (binding domain and activation domain come together). Whatman #5 filters (VWR) were placed over 150 mm plates, filters were marked and yeast colonies partially transferred. Filters were submerged in liquid nitrogen for 10 seconds, thawed at room temperature and then placed onto pre-soaked filters containing X-Gal substrate and incubated at room temperature for 30 minutes. The X-Gal substrate included 5 mls Z Buffer/X-Gal solution; 100 mls Z Buffer (Z Buffer: 16.1 g/L $Na_2HPO_4 7H20$, 5.5 g/L $NaH_2PO_4 H_2O$, 0.75 g/L KCL, 0.246 g/L $MgSO_4 7H_20$, pH7), 0.27 mls β-mercaptoethanol (β-ME, Sigma) and 1.67 mls X-Gal stock solution (20 mg/ml of 5-bromo-4-chloro-3-indolyl-β-D-

galactopyranoside (X-GAL, Sigma)), dissolved in N, N-dimethylformamide (DMF)). The presence of blue colonies indicated LacZ gene activity.

Mating Assay

After rigorous determination of true protein-protein interactions (selection and activity of reporter genes), potential positive yeast clones were isolated for further testing in a yeast mating assay. The positive yeast clones were resuspended in 200 µl dH_2O and vortexed briefly. The cell suspension (100 µl) was plated on SD/-Leu/cycloheximide (10 µg/ml) and grown at 30°C for five days. The Y190 yeast cell line becomes cycloheximide sensitive in the presence of the pAS2-1 plasmid. The surviving yeast do not contain pAS2-1 plasmid (only the pGAD10 plasmid carrying positive library clone survive) which was confirmed by loss of growth on SD/-Trp plates. The Y187 yeast cell line was transformed with control plasmids (pAS2-1 alone and pGAD10 alone) as well as with pAS2-1/Bait of interest. The two yeast strains (Y190 and Y187) were mated by placing one colony of each type in 500 µl YPD medium (20 g/L Difco peptone, 10 g/L Yeast extract, 18 g/L Agar (for plates) pH 5.8, 2% dextrose), vortexed and incubated at 30°C with shaking (200 rpm) overnight. The mating culture was spread onto 100 mm plates containing SD/-Leu/-Trp and SD/-Leu/-Trp/-His/+3-AT plates. Plates were incubated at 30°C for five days and phenotypes examined. The presence of LacZ and HIS3 positive phenotypes confirmed a positive protein-protein interaction.

DNA Isolation from Yeast

To isolate the library clone from yeast, a 5 ml culture was grown in SD/-Leu/-Trp/-His/3-AT media overnight at 30°C with shaking at 230 rpm. Cultures were centrifuged and the pellet resuspended in 200 µl of yeast lysis solution (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM

Tris (pH 8.0), 1 mM EDTA) followed by adding 200 µl phenol/chloroform/isoamyl alcohol (25:24:1). Acid-washed glass beads (0.3 g, Sigma) were then added and samples vortexed for approximately two minutes. Samples were centrifuged at 14,000 rpm for five minutes at room temperature and DNA isolated by EtOH precipitation with two volumes of 95% EtOH and 1/10 volume of 3 M sodium acetate (NH_4OAc) pH 5.2. Pellets were washed with 70% ethanol, dried under vacuum and then resuspended in 20 µl TE. Restriction digestion with Eco RI verified the presence and size of an insert. Library clones were also transformed (by heat shock) into *Escherichia coli* (E. coli) DH5α cells (Gibco BRL) and the DNA sequence determined using the pGAD10 forward oligonucleotide. DNA sequences were translated to amino acid sequences using ExPASy (http://www.expasy.org/tools/dna.html) and BLAST searches were performed at NCBI (http://www.ncbi.nlm.nih.gov/BLAST/) to identify proteins.

Isolation of Rat Nell2 cDNA

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

HG87 forward oligonucleotide and HG90 reverse oligonucleotide were used to amplify the full length Nell2. A 5' Eco RI site and a 3' Not I restriction site were added to the primers. HG87 was designed using the PKC-BP Nell2 sequence (GenBank Accession U48245). The HG90 oligonucleotide was based on the sequence from the isolated yeast two-hybrid positive clones. RT was carried using whole rat brain RNA (see below) and PCR performed by a "touch up" program ranging from 33°C to 41°C. The 2448 bp coding region was subcloned into the pGem-T Easy T-tailed plasmid (Invitrogen) and the DNA sequence confirmed with internal oligonucleotides (**Appendix Figure 5**). Nell2 was further subcloned into pcDNA3-1 (Invitrogen). A FLAG epitope (Su et al., 1992) was added to the N-terminus of the Nell2 via PCR using forward oligonucleotide HG35 and reverse oligonucleotide HG36 whereby a 317 bp PCR product was generated, subcloned into pGEM-T Easy (Invitrogen) and sequenced. The

FLAG/Nell2 full-length construct was cloned into pcDNA3-1. DNA sequence was confirmed in pcDNA3-1 using the T7 forward primer.

Antisense and Deletion Mutant Nell2 Clones

An antisense Nell2 construct was made using 198 bp of the 5' sequence. The forward oligonucleotide HG87 contains an Eco RI site and HG85 contained a Hind III restriction site. PCR was performed by seven cycles at a 41°C annealing temperature, followed by a 65°C annealing temperature for 25 cycles. The PCR product was subcloned into the pGem-T Easy T tailed cloning plasmid and sequenced using T7, SP6, HG87 and HG85 primers. The clone was inserted 3' to 5' into pcDNA3-1 and the sequence confirmed using the T7 forward primer.

Deletion Mutant A (-1 to 248 aa) was constructed using oligonucleotide HG40 and reverse oligonucleotide HG90 generating a 1680 bp PCR which was then subcloned into pGEM-T Easy, sequenced and further subcloned into pcDNA3-1. Deletion Mutant B (-455 to 649 aa) involved HG87 (which included an Eco RI restriction site and a Flag epitope) and reverse oligonucleotide HG43 to generate a Flag-tagged 1320 bp PCR product. Subsequently, a 480 bp PCR product was generated from the HG44 forward oligonucleotide and HG90 and the 1320 bp and 480 bp fragments were ligated together to form a full-length cDNA. The full-length product was subcloned into pGEM-T Easy, sequenced and then further subcloned into pcDNA3-1. Deletion Mutant C (-661 to 816 aa) was generated from oligonucleotides HG87 and HG45 giving a Flag-tagged 1980 bp PCR product. The product was subcloned into pGEM-T Easy, sequenced and then further subcloned into the pcDNA3-1 plasmid. Deletion Mutant D (-1 to 101 aa) was amplified with oligonucleotides HG71 (containing an Eco RI restriction site and a Flag epitope) and HG72 generating a Flag-tagged 795 bp PCR product which was cloned back into the original pcDNA3/Nell2 clone at the BsM I/Eco RI restriction enzyme sites. Deletion Mutant E (-1 to 20

aa) was generated with HG76 (containing an Eco RI restriction site and a Flag epitope) and HG77 giving a 234 bp PCR product which was cloned back into the original pcDNA3/Nell2 clone at the BstE II/Eco RI restriction enzyme sites. All PCR products were generated using a touch down protocol at an annealing temperature 5°C below the complementary TM of the oligonucleotide.

RNA Expression Studies

RNA Extraction From Cell Lines

Total RNA was isolated from two human embryonic kidney (HEK) cell lines. The 108 HEK cell line stably expresses N-type calcium channels (α_{1B}, β_{1b} and $\alpha_2\delta$ subunits, Bourinet et al., 1996, Stea et al., 1995). The 201 HEK cell line represents the native HEK293 cell parental line with the addition of the simian virus 40 (SV40) large-tumor (T) antigen which increases plasmid promoter activity (Chang et al., 1985, Lebkowski et al., 1985). One 100 mm plate containing a 10 ml cell culture was centrifuged and the pellet resuspended in 1 ml extraction buffer (4 M Guanidinium Isothiocynate, 20 mM Na-acetate (pH 5.2), 0.1 mM DTT, 0.5% N-Lauroylsarcosine) and then incubated on ice. Twenty µl of β-mercaptoethanol was added and the sample mixed by pipetting. Na-acetate (100 µl, 2M, pH 4.0) was added and the sample mixed gently. H_2O-saturated phenol (1 ml) was added and then mixed. Finally, chloroform (200 µl) was added gently, mixed, incubated on ice 20 minutes and then centrifuged for 10 minutes. The supernatant was precipitated with 100% EtOH for two hours and the pellet washed three times with 80% EtOH. The pellet was resuspended in 20 µl TE, 1 µl FPLC pure DNase I and then incubated at 37°C for 30 minutes followed by incubation at 80°C for 10 minutes to inactivate the DNase I. RNA was precipitated with 95% EtOH and 1/10 volume 3 M Na-acetate. Samples were quantified by UV spectroscopy at A_{260}.

RNA Extraction From Rat Tissues

One adult male and one adult female rat were sacrificed using chlorohydrate (150 mg/kg) and various tissues collected (adrenal gland, brain stem, cerebellum, cortex, eye, heart, kidney, liver, ovary, pituitary, seminal vesicle, and spleen). Tissue samples (50-100 mg) were homogenized in 1 ml TRIZOL Reagent (BRL) and incubated at room temperature for five minutes. One ml of chloroform was added and samples were vortexed, incubated at room temperature for three minutes and then centrifuged at 12,000 X g for 15 minutes at 4°C. The aqueous layer was collected and 500 µl of isopropyl alcohol was added. Samples were incubated at room temperature for 10 minutes, centrifuged at 12,000 X g for 10 minutes at 4°C and the pellet was washed with 75% EtOH (1:1 ratio with TRIZOL). Samples were mixed by vortexing and centrifuged at 7,000 X g at 4°C for five minutes. The pellet was dried for 5 – 10 minutes and resuspended in RNase-free dH_2O. Samples were incubated at 55°C for 10 minutes to ensure that the RNA was in solution. RNA concentration was determined by spectroscopy at A_{260}.

RT-PCR

i. Tubulin

As a positive control for RT-PCR, tubulin was amplified from each rat tissue RNA sample and HEK cell line. Oligonucleotides (JM91 forward oligonucleotide and JM92 reverse oligonucleotide) were designed against the rat α-tubulin sequence from GenBank (Accession V01227, Wisden, 1994) to amplify a 223 bp PCR product. An oligonucleotide (JM93) internal to the PCR fragment was radiolabeled with $\gamma^{32}P$ and used on a Southern blot to confirm presence of tubulin. In addition, in one case the tubulin RT-PCR product from cerebellum was isolated, subcloned into pGEM-T Easy and the DNA sequence confirmed with the T7 forward oligonucleotide. Negative control samples had no RNA in the RT-PCR reaction.

ii. α_{1B} Subunit

RT-PCR was carried out using oligonucleotides (HG103 and HG105) generated against the 3' end of the carboxyl terminus of the α_{1B} subunit. A 417 bp PCR product was amplified from various rat tissues and some HEK cell lines. A Southern blot was performed using an internal oligonucleotide to the region amplified. The positive control contained α_{1B} subunit cDNA template in the PCR mix and the negative control was RT-PCR in the absence of RNA.

iii. Nell2

In order to determine the expression of Nell2 mRNA in various tissues of adult rat, forward oligonucleotide HG28 and reverse oligonucleotide HG29 (against the 3' non-coding region of the Nell2) were used to generate a 314 bp PCR product using RT-PCR. A Southern blot was carried out using radiolabeled forward oligonucleotide HG30 which binds internally to the PCR product. In addition, the cerebellum RT-PCR product was isolated, subcloned into pGEM-T Easy and the DNA sequence determined to confirm the Nell2 non-coding region. Forward oligonucleotide HG99 and reverse oligonucleotide HG90 were used to amplify a 590 bp fragment via RT-PCR using RNA extracted from both 108 and 201 HEK cells. γ^{32}P radiolabeled HG84 forward oligonucleotide, which binds to an internal region of the PCR product, was used as a probe for Southern blotting. RT-PCR products from both cell lines were isolated, subcloned into pGEM-T Easy and the DNA sequence determined to confirm the identification of the Nell2 sequence.

Southern Blotting

The RT-PCR products were run on a 1% agarose gel which was incubated in 0.25 M HCl for 15 minutes, rinsed in dH_2O, soaked in 0.5 M NaOH/1.5 M NaCl 45 minutes, rinsed in dH_2O, soaked in 1 M NH_4OAc for 30 minutes and then blotted to treated Nytran+ (Amersham). Nytran+ was

treated by first wetting in dH_2O and then soaking in 1 M NH_4OAc for 15 minutes. The agarose gel was placed between two pieces of Nytran+ and 3 M Whatman paper and paper towels. DNA was transferred overnight at room temperature, the blot was dried at 37°C and then exposed to radiolabeled oligonucleotide for blot hybridization.

Blot Hybridization

For colony screening, colonies were grown overnight at 37°C on Hybond-N nytran filters (Amersham) and then pretreated before hybridization (incubated two minutes 0.5 M NaOH, five minutes 1 M Tris (pH 7.4), five minutes 1.5 M NaCl/0.5 M Tris (pH 7.4)). Filters were then microwaved at high power for two minutes to fix DNA. Filters were pre-hybridized in hybridization buffer for 30 minutes (5 X SSPE (1 L at 20 X; 175.3 g NaCl, 27.6 g $NaH_2PO_4 \cdot H2O$, 7.4 g EDTA, pH 6.8), 0.3% SDS, 2.5 X Denhardt's (50 X; 5 g of Ficoll (Type 400, Sigma), 5 g of polyvinylpyrolidone, 5 g of bovine serum albumin (Fraction V, Sigma) in 500 mls dH_2O)) prior to adding probe. For random primed probes; 25 ng of DNA was boiled in a total volume of 20 µl for five minutes, cooled on ice, and 15 µl of random primer buffer (Amersham) added together with 2 µl dNTPs (dTTP, dGTP and dCTP) 5 µl of [α^{32}P]dATP (10 mCi/ml, Amersham), 1 µl Klenow DNA polymerase (BRL) and the volume topped up to 50 µl. In some cases [α^{32}P]dCTP (10 mCi/ml, Amersham) was also included to increase signal detection. Samples were incubated at room temperature two hours and unincorporated α^{32}P was removed by separation through a G-25 spin column (Pharmacia). For oligonucleotide end labeling, 7 pmol oligonucleotide was added to 4 µl kinase buffer (BRL), 2 µl γ^{32}P ATP (10 mCi/ml, Amersham), 1 µl T4 Kinase (BRL) and to a final volume of 20 µl with dH_2O. Samples were incubated at 37°C for two hours, and then passed through a G-25 spin column, heated to 65°C for five minutes and placed on ice before adding to pre-hybridization buffer. Filters were

hybridized overnight (usually at 63°C for oligonucleotide probes under 30 bp or 5°C below the calculated TM for DNA probes greater than 30 bp) in a water bath with mild agitation. After hybridization filters were washed three to five times in 1X SSPE, air dried and exposed to X-ray film (Kodak XAR-5).

Generation of Polyclonal Antisera Against Nell2

GST Fusion Construction

Rabbit polyclonal Nell2 antisera was generated against a Nell2 glutathione S-transferase (GST) fusion protein. The forward oligonucleotide HG33 and reverse oligonucleotide HG34 were used to generate a 261 bp PCR product which was then subcloned into pGEM-T Easy. The Nell2 DNA sequence was confirmed with T7 forward and SP6 reverse oligonucleotides. The PCR product was further subcloned into pGex4T-1 and the sequence confirmed with the pGex4T-1 forward oligonucleotide.

To determine if the GST fusion of Nell2 resulted in the correct size protein, a 2 ml culture of the construct in bacterial cells was grown overnight at 37°C with shaking in 2XYT (16 g bacto-tryptone, 10 g bacto-yeast extract, 5 g NaCl up to 1 L with dH_2O) with ampicillin (50 μg/ml). A 10 ml culture was inoculated and after 1.5 hours the culture density was measured with UV spectroscopy at 600 λ to readings between 0.4 to 0.6. A 1 ml sample was collected (non-induced) and 16 μl of 1 M Isopropylthio-β-D-galactoside (IPTG, Gibco BRL) was added to the remaining culture which was then incubated at 37°C with shaking for three hours. A 1 ml sample was collected and centrifuged for 10 minutes, the pellet was resuspended in 50 μl of 2 X SDS sample buffer and boiled five minutes. Two μl samples were loaded onto a 12% SDS-PAGE to compare non-induced and induced proteins by Commassie staining. The GST protein was 26 kDa and the

Nell2 fusion fragment was 9.6 kDa giving a GST fusion protein product of 35.6 kDa.

Purification of Protein Antigen

Once the fusion protein size was confirmed, large scale protein purification was carried out as described (Smith and Johnson, 1988). A 10 ml culture of E. coli transfected with the GST fusion protein was grown overnight at 37°C with shaking and was used to inoculate a 250 ml culture in 2XYT with ampicillin. The culture was grown for three hours (until 600 λ was 0.4 – 0.6) and 400 μl IPTG was added. The culture was grown for three hours, centrifuged and the pellet resuspended in MT-PBS (0.5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF)). Cells were lysed by sonication (6 times for 30 seconds) on ice and 1 ml of 10% Triton X-100 in MT-PBS added. The sample was then mixed at 4°C with shaking for five minutes, centrifuged 10 minutes and the supernatant collected. Glutathione agarose beads were pre-swollen and washed two times in MT-PBS/1% Triton X-100. Beads were added to the supernatant and mixed at room temperature for five minutes. The beads were then centrifuged and washed five times in 15 mls of MT-PBS/1% Triton X-100. Beads were then washed two times in 15 mls of MT-PBS. Elution was carried out three times with five minute intervals by incubating beads with 2-3 mls 50 mM Tris-HCL, 10 mM glutathione. Fractions were stored at –20°C. Protein concentration was measured at A_{280}.

Rabbit Polyclonal Nell2 Antisera

Two New Zealand white female rabbits (BP1 and BP2), housed at UBC Animal Care South Campus, were each initially injected with purified fusion protein (100 mg) using Freund's complete adjuvant. Rabbits were boosted at four week intervals with the fusion protein (100 mg) diluted in Freund's incomplete adjuvant. Blood samples were taken 12 days post injection and

full body bleeds after six boosts. Antibody immunogenic response was monitored by Western blots using purified fusion protein comparing pre-immune and immune serum.

Purification of Anti-Nell2 Antisera

Nell2 antibodies were purified by cyanogen bromide (CNBr)-affinity chromatography as described (Harlow, 1988). Briefly, two columns were prepared using CNBr-activated Sepharose 4 fast flow (Amersham), one using GST protein and the other using GST/Nell2 fusion protein (Nell2 antibody antigen). Proteins underwent dialysis in coupling buffer (0.1 M $NaHCO_3$ (pH 8.3), 0.5 M NaCl) 4°C overnight. Columns were prepared by pre-swelling and washing beads in 1 mM HCl. Initially, antibody crude serum (10 mg) was added to GST/Nell2 antigen affinity column first, incubated overnight at 4°C and then washed with 25 mM Tris (pH 7.5)/0.02% azide. A final wash of 25 mM Tris (pH 7.5)/500 mM NaCl was carried out. Elution was performed with an acidic solution (100 mM glycine (pH 2.4)), washed with 10 mM Tris (pH 7.5)/500 mM NaCl followed by an elution with a basic solution (100 mM triethylamine, pH 11.8). The same procedure was repeated with the eluted antibody sera using a GST affinity column. Samples were taken before (pre-column) and after (post-column) each column elution procedure. Eluted antibody was equilibrated to 0.1 M Tris (pH 8.0) and concentrations determined by A_{280}. Comparisons were made between pre-column and post-column antibodies by Western blot analysis.

Western blot Analysis

SDS-PAGE was carried out on protein samples denatured in sample buffer. Protein size was determined by comparison to pre-stained standard high molecular weight markers (BRL). Protein quantification was carried out by UV spectroscopy (A_{280}, 1 absorbance unit = 1 mg/ml protein) or by Modified Lowry Assay (Peterson, 1977, Markwell et al., 1978). Either 8%, 10% or 12%

polyacrylamide gels were prepared as described in Harlow, 1988. Gels were run at 100 volts for two hours in running buffer (0.25 M Tris base, 1.5 M glycine and 0.1% SDS) using a mini-gel apparatus (BioRad). Polyacrylamide gels were soaked in transfer buffer (48 mM Tris base, 39 mM glycine, 0.37% SDS and 20% methanol) for 10 minutes and proteins transferred to Nytran+ (Amersham) using a semi-dry electrophoretic transfer apparatus system (BioRad) at 10 volts for two hours. The membranes were then blocked for 1 hour to overnight at 4°C with shaking in blocking solution (TBS (25 mM Tris, 0.8% NaCl, 0.02% KCl, (pH 7.6))/10% dry milk powder (Carnation)). Membranes were incubated in primary antibody in TBS with 5 to 10% dry milk powder for four hours to overnight at 4°C with shaking. Membranes were washed three times in TBST (TBS/1% Tween-20 (Sigma)) at 4°C with shaking and then incubated at 4°C with shaking for 1 hour to overnight in species-specific horse radish peroxidase-linked secondary antibody (Amersham) in TBS containing 5% to 10% dry milk powder. Membranes were then washed five times in TBST at 4°C for 10 minutes with shaking. ECL chemiluminesence was performed by incubating membranes in 2 mls of ECL kit reagents (Amersham) at room temperature for 1 minute and exposing the blots to X-ray film (Biomax MS, double emulsion film from Kodak) for 1 minute to overnight.

Antibodies used included the anti-FLAG M5 mouse monoclonal antibody which is specific for an N-terminal Met-FLAG fusion protein (N-terminal M-D-Y-L-D-D-D-D-L, Sigma). For Western blots, 20 μg/ml was used. The anti-Nell2 rabbit polyclonal was used at a 1/500 dilution of crude serum. Both anti-rabbit and anti-mouse secondary horse radish peroxidase antibodies were used as recommended (1/5000, Amersham).

HEK Cell Expressed Protein

DNA Transfection of HEK Cells and Neurons

i. $CaPO_4$ Transfection

For a 100 ml tissue culture plate at 80% confluency, 20 μg total DNA was added to 500 μl of 250 mM $CaCl_2$ and added slowly to 500 μl 2 X HeBS (274 mM NaCl, 40 mM Hepes, 12 mM Dextrose, 10 mM KCl and 1.4 mM Na_2HPO_4, (pH 7.05)), as previously described (Chen and Okayama, 1987). After incubation for 20 minutes at room temperature, the media was removed and the mixture was dribbled over cells which were then incubated overnight at 37°C/CO_2. The media was changed 12 to 24 hours postransfection. Total DNA included carrier DNA (50:50 ratio) usually pGem-T Easy plasmid. Media for HEK cells was Dulbecco's Modified Eagle's media (DMEM, BRL), supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin at 100 μg/ml (BRL).

ii. Lipofectamine

Cells were grown to 80% confluency at 37°C/CO_2. For a 100 mm plate, 12 μg of DNA was diluted into 1 ml serum free media, OPTI-MEM (Gibco BRL), 30 μl Lipofectamine Reagent (Gibco BRL, Hawley-Nelson, 1993) was diluted into 1 ml serum free media and mixed with DNA solution for 45 minutes at room temperature. Serum free media (8 mls) was added to transfection mixture, cells were rinsed with serum free media and transfection media was added to cells for incubation for 12 hours at 37°C/CO_2. Media was then replaced with DMEM/10% FBS/penicillin/streptomycin (100 μg/ml).

iii. Particle-mediated Gene Transfer

Primary cortical neurons from male and female Wistar rats embryonic day 18 (e18) were dissociated at high density (1.5 million cells/ml) and cell cultures were grown in 35 mm well

plates (Mackenzie et al., 1996). Four days post plating, particle-mediated gene transfer was carried out using BioRad Helio Gene-Gun as per protocol (Lo et al., 1994). The particle size was 0.6 μm and the pressure was at 140 psi. Cell staining was done two days post transfection.

Protein Isolation From HEK Cells

HEK cells (108 or 201 cell lines) were transiently transfected utilizing the $CaPO_4$ method or by Lipofectamine as previously described. Two days post transfection, cells were lysed in lysis buffer (150 mM NaCl, 1% NP-40, 50 mM Tris pH 7.6, 1 mM EDTA, 0.5% Deoxycholate, 0.1% SDS, Protease Inhibitor Cocktail (Boehringer Mannheim)) and incubated on ice for 1 hour. The lysed cells were collected with a rubber spatula, centrifuged at 4°C for 15 minutes and the supernatant was ultra centrifuged at 100,000 X g, 1 hour at 4°C. Supernatant (cytosolic and secreted proteins) was separated from pellet (membrane proteins). Membrane proteins were resuspended in sample buffer (without DTT and BPB) and protein concentration was determined by Modified Lowry Assay (Peterson, 1977, Markwell et al., 1978).

In vitro Binding Assays

***In vitro* Translation**

The TNT Rabbit Reticulocyte Lysate System (Promega) was used to generate radiolabeled proteins. A typical 50 μl reaction using [^{35}S]-methionine contained 25 μl TNT Rabbit Reticulocyte Lysate, 2 μl TNT reaction buffer, 1 μl TNT RNA T7 polymerase, 1 μl amino acid mixture (all amino acids at 1 mM except methionine), 2 μl [^{35}S] methionine (100 Ci/mmol at 10 mCi/ml, Amersham), 1 μl RNasin Ribonuclease Inhibitor (40 U/μl, BRL), 2 μl DNA template (0.5 μg/μl) to 50 μl with nuclease free dH_2O. The reaction was incubated at 30°C for 90 minutes. Reactions were sometimes put through a G-25 spin column before use in order to

remove unincorporated nucleotides.

Ni^{2+} Pull Down Assay

Ni^{2+}-NTA resin (Pharmacia) was washed three times in hybridization buffer (200 µl beads/10 mls buffer) by shaking at 4°C with mild centrifugation between washes. Usually 20 to 40 µl prepared beads were used per pull down experiment. [^{35}S]-methionine labeled *in vitro* translated proteins were bound to pre-washed Ni^{2+}-NTA in hybridization buffer (50 mM NaH_2PO_4, 300 mM NaCl and 20 mM imidazole (pH 8)) and eluted with various concentrations of imidazole and different pH (pH 8 to pH 5 or 20 mM to 240 mM imidazole in 100 mM $CaCl_2$, 100 mM $NaCl_2$, 50 mM Hepes, 5% BSA, PBS, (125 mM NaCl, 3 mM KCl, 5 mM Na_2HPO_4, 2 mM KH_2PO_4 (pH 7.8)). In initial experiments, it was determined that 60 mM imidazole or pH 7.7 buffer eluted Bait 4 (*in vitro* translated in PET plasmid containing His tag) but not Nell2. Several pull down experiments were also carried out using 80 mM imidazole for elution.

Co-localization Studies

Immunocytochemistry in HEK Cells and Neurons

Transiently transfected HEK cells (201 or 108) via standard $CaPO_4$ transfection or Lipofectamine were grown in 35 mm petri dishes on glass cover slips (No. 2, Fisher). Glass coverslips were acid-etched (boiled for 10 minutes in 0.1 M HCl, rinsed three times in dH_2O and stored in 95% EtOH) and flamed before use. Transfection of N-type channels included α_{1B}, or green fluorescence protein (GFP)/N-type (α_{1B}/GFP), $\alpha_2\delta$ and β_{1b}. GFP/N-type is the α_{1B} subunit fused to the GFP at the C-terminus, a generous donation from Kurt Beam (Grabner et al., 1998). Nell2 cDNA was either full length, Flag-tagged on the N-terminus or Deletion Mutant E (-1 to 20 amino acids of Nell2). The control cDNA used was the pGEM-T Easy plasmid. The media was removed 1 to two days post-transfection and cover slips were washed with PBS. Cells grown

on cover slips were incubated for 10 minutes in 3% para-formaldehyde (1 ml/plate) at room temperature (fix). Coverslips were washed three times in PBS. Coverslips were incubated for two minutes (1 ml/plate) with 100% MeOH (permeablize) at room temperature. Coverslips were washed five times with PBS for two minutes at room temperature, coverslips were placed on parafilm and incubated in a humidified chamber with 100 µl primary antibody solution (TBS/1% dry milk powder) overnight at room temperature. M5, mouse monoclonal anti-FLAG antibody (Sigma) was used at 20 µg/ml. Affinity purified anti-α_{1B} rabbit polyclonal antibody (generated by Don Nelson) was used at 5 µg/ml. Anti-Nell2 rabbit polyclonal antibody (pre-immune and immune sera) was used at 1/1000 crude lysate. For protein inhibition experiments, 10 mM fusion protein was used in the primary antibody solution. Coverslips were washed three times for five minutes in wash solution (PBS/1% Triton-X 100 (Sigma)). Coverslips were incubated in secondary antibody solution (TBS/5% dry milk containing 0.025 ng 4',6'-dimidino-2-phenylindole (DAPI)) in 500 µl/coverslip at room temperature for two hours in humidified chamber. DAPI, a DNA-intercalating agent, allows detection of the nucleus of cells at a different wavelength than fluorescein or Texas red. Fluorescein (FITC)-conjugated anti-rabbit IgG secondary, Rhodamine conjugated to anti-rabbit IgG and Texas Red dye-conjugated anti-mouse IgG secondary (Jackson ImmunoResearch Laboratories, Inc.) were used at 1/200 dilutions. Coverslips were washed five times with wash solution for five minutes and mounted on slides with mounting media (2.5% 1,4-diazobicyclo-[2.2.2]-octane (DABCO) in glycerol). DABCO was used as a bleaching retardant for fluorescein. Cells were visualized and photographed using a fluorescent microscope (Dr. Don Moerman's Laboratory). DAPI excites at 365 nm emitting blue color, fluorescein excites at 495 nm emitting a green color and Texas red excites at 596 nm emitting a red color.

Primary cortical neurons were grown 16 to 25 days on poly-D-lysine-coated 12 mm glass

coverslips slips in 35 mm plates at high density as described (Mackenzie et al., 1996). Cells were transfected by particle-mediated gene transfer with Flag-tagged Nell2, stained with mouse M5 anti-Flag monoclonal antibody, secondary anti-mouse conjugated to Texas Red and DAPI. Imaging was performed on a BioRad MRC 600 confocal microscope (Dr. Tim Murphy's laboratory). Magnification was at 63 X oil-immersion Zeiss lens. Vertical z-scanning was used. For three-dimensional reconstruction of cells, a maximal intensity projection was employed.

Electrophysiological Recording from Transfected HEK Cells

Modulation by Protein Kinase C

Whole cell patch clamp recordings were carried out on human embryonic kidney (HEK) cells stably expressing rat brain N-type channels (performed by Arik Hasson). Cells were split and plated at 10 to 20% confluency on 35 mm culture dishes in DMEM/10% FBS/penicillin/streptomycin (100 μg/ml) and incubated at 37°C. Individual 35 mm culture dishes were filled with external recording solution and subsequently perforated whole-cell patch-clamp recordings were performed using an Axopatch 200A amplifier linked to a personal computer.

The bath potential was nulled to ground by means of a 3 M KCl Agar bridge. Borosilicate patch pipettes showed typical resistances of 2.5 to 3.5 MΩ after being filled with internal solution and 0.1% (W/V) nystatin. The internal pipette solution contained (in mM): 65 Cs Gluconate, 40 CsCl, 25 TEACl, 1 $CaCl_2$, 11 EGTA, 10 Hepes (pH 7.2). The external recording solution contained 20 $BaCl_2$, 1 $MgCl_2$, 10 HEPES, 25 TEACl, 10 Glucose, (87.5) CsCl, (pH 7.3). On average, two to five minutes after GΩ seal was formed, nystatin produced a series resistance of 20MΩ or less, that was compensated by 0 to 95% by the Axopatch instrument. Under these conditions, the amount of N-type calcium current "run-down" was less than 5% over 30

minutes. Currents were typically elicited from a holding potential of -100 mV to various test potentials using Clampex. Data were filtered at 2 or 5 kHz and recorded directly onto the hard drive of the computer. Current leak components were digitally subtracted using the P/4 capability of the data acquisition software.

The drugs for electrophysiological experiments were initially dissolved in DMSO (apart of BAPTA-AM) and then dissolved in the external recording solution and applied at the designated concentrations together with 1 to 2 mg/ml (final concentration) of BSA. DMSO concentration in the recording chamber was always less than 1%. The phorbol esters, 12-myristate-13-acetate (PMA) and phorbol 12, 13-dibutyrate (4β-PDBu) were dissolved in DMSO, protected from light and were stored as stock solutions of 1 to 10 μM in a -20°C freezer. Drugs were applied by bath application. The drug concentrations indicated relate to the final estimated drug concentration in the experimental chamber.

Modulation by G-proteins

HEK cells were transfected with N-type calcium channel subunits α_{1B}, $\alpha_2\delta$ and β_{1b} at a 1:1:1 molar ratio, using a standard calcium phosphate protocol as described (Chen and Okayama, 1987). After 12 hrs, the medium was replaced with fresh DMEM. The cells were allowed to recover for additional 12 hrs and subsequently removed to a 28°C CO_2 incubator for two days prior to recording.

Whole-cell patch-clamp recordings (membrane ruptured) were performed using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA) linked to a personal computer equipped with pClamp v7.0. Patch pipettes (Sutter borosilicate glass, BF 150-86-15) were pulled using a Sutter P-87 microelectrode puller, fire polished using a Narashige microforge. Pipettes were

filled with internal solution containing 108 mM Cs-methanesulfonate, 4 mM $MgCl_2$, 9 mM EGTA, 9 mM HEPES (pH 7.2 adjusted with TEAOH). Pipettes with resistance in the range of 2-4 mΩ were used in this study. The cells were transferred to a 3 cm culture dish containing recording solution comprised of 20 mM BaCl, 1 mM $MgCl_2$, 10 mM HEPES, 40 mM TEACl, 10 mM glucose, 87.5 mM CsCl (pH 7.2 adjusted with TEAOH). After obtaining a gigaohm-seal, cell capacitance and the series resistance were compensated. Currents were elicited from a holding potential of -100 mV to various test potentials generated using Clampex software (Axon Instruments), filtered at 1 kHz using a 4 pole Bessel filter, and digitized at a sampling frequency of 2 kHz.

A standard double pulse protocol was used in these experiments. Specifically, a 50 ms depolarizing prepulse from a holding membrane potential of -100 mV to 150 mV was followed by a 100 ms test pulse to 20 mV. The prepulse and test pulse were separated by a 5 ms step return to -100 mV. Data were analyzed using Clampfit (Axon Instruments). All curve fittings were carried out in Sigmaplot 4.0 (Jandel Scientific).

Statistics

Data were presented as the mean ± SE format. Statistic analysis was carried out using SigmaStat 2.0 (Jandel Scientific). Differences between mean values from each experimental group were tested using either a Student t-test for two groups or one way analysis of variance (ANOVA) for multiple comparisons. Differences were considered significant if $p < 0.05$.

Chapter 3. Isolation of Nell2, a Protein Kinase C Binding Protein Which Interacts with the N-type Calcium Channel.

Background

The modulation of some calcium channel properties such as channel kinetics and membrane targeting have been attributed to the calcium channel auxiliary subunits. While these interactions have lead to a better understanding of N-type channel structure-function, the further identification of novel protein interactions should help elucidate regulatory mechanisms involved in their multiple functions, including neurotransmission, at the molecular level.

The first N-type calcium channel protein-protein interaction identified was between the α_{1B} subunit and syntaxin-1A, a synaptic vesicle release protein (Sheng et al., 1994, Bezprozvanny et al., 1995, Mochida et al., 1996, Rettig et al., 1996, Sheng et al., 1996, Sheng et al., 1997). This finding subsequently lead to the identification of the α_{1B} subunit "Synprint" site (residues 718 to 963 in the rat α_{1B} subunit), which binds several of the soluble N-ethylmaleimide-sensitive attachment factor receptor (SNARE) proteins; syntaxin-1A, SNAP-25 and synaptotagmin (Leveque et al., 1992, Leveque et al., 1994, Sheng et al., 1994, Sheng et al., 1996). N-type channels interact with the SNARE proteins in a calcium-dependent manner *in vitro*, providing a model of regulation for neurotransmission *in vivo* (McEnery et al., 1998). The docked vesicle proteins syntaxin-1A and SNAP-25 form a complex with the α_{1B} subunit at low calcium

concentrations (<10 μM). As calcium concentration increases (10 to 20 μM), further stabilization of the interaction occurs. When the threshold of calcium reaches 20 to 50 μM range binding affinity becomes reduced and syntaxin-1A and SNAP-25 dissociate from the channel. Binding of syntaxin-1A to synaptotagmin occurs at higher calcium concentrations (100 μM to 1 mM, Chapman et al., 1995; Li et al., 1995a; Li et al., 1995b, Kee and Scheller, 1996). Synaptotagmin has been identified as the calcium sensor, binding syntaxin-1A at 10 to 50 μM and to phsopholipids at 100 to 300 μM *in vitro* (Perin et al., 1990, Brose et al., 1992, Davletov and Sudhof, 1993, Chapman and Jahn, 1994, Geppert et al., 1994). Taken together, the biochemical evidence suggests N-type calcium channels can play a direct role in neurotransmission by not only supplying the required threshold intracellular calcium concentration, but initiating priming, docking and fusion of vesicles for exocytosis.

A further functional complexity of the N-type channel/SNARE protein interaction occurs in that phosphorylation of the α_{1B} subunit Synprint site by PKC or CaMKII (but not PKA or PKG), strongly inhibits binding of syntaxin-1A or SNAP-25 to the channel. In contrast, phosphorylation of syntaxin-1A and SNAP-25 with PKC and CaMKII does not attenuate binding interactions with the Synprint site, suggesting additional possible mechanisms of regulation of neurotransmitter release (Yokoyama et al., 1997). In both HEK cells and chick sensory neurons, syntaxin-1A co-expression has also been shown to modulate N-type channel currents by acting through certain G-proteins (Jarvis and Zamponi, 2001, Lu et al., 2001).

Other protein-protein interactions that play a role in exocytosis involving N-type calcium channels have been identified. For example, the human and rat N-type α_{1B} subunits have been found to associate with a tripartite complex consisting of modular adapter proteins CASK, Mint

1 and Veli (Maximov et al., 1999). The PDZ domain of rat Mint 1 associates to a potential PDZ binding domain within carboxyl terminus of the α_{1B} subunit. Additionally, the α_{1B} subunit interacts with the CASK SH3 domain in a proline rich region of the C-terminus as well. Mint and CASK have been shown to bind strongly within the tripartite complex (Butz et al., 1998). The significance of this finding has led to a model whereby the interaction of modular adaptor proteins/N-type calcium channel presynaptic complexes are physically associated to NMDA receptors and potassium channels via β-neurexin/neuroligin interaction (Ichtchenko et al., 1995, Nguyen and Sudhof, 1997) at postsynaptic sites. Tight association of this PDZ network of interacting proteins may contribute to the efficient, rapid neurotransmission (Schneider et al., 1994).

Other complexes involving protein networks that affect calcium channels and that also function through PDZ domains are the A kinase anchoring proteins (AKAPs). AKAPs participate in ion channel modulation, playing a role in localization of upstream activators and downstream targets of calcium channels, NMDA/kainate glutamate receptors and potassium channels (Fraser and Scott, 1999, Dodge and Scott, 2000). The well characterized AKAP79 acts as a scaffold for at least three proteins; PKA, calcineurin and PKC (Coghlan et al., 1995, Klauck et al., 1996). It has been determined through immuno-copurification processes that AKAP15 associates with the α_{1S} subunit of L-type skeletal muscle calcium channels (Gray et al., 1997, Gray et al., 1998). Cloned cardiac L-type channel subunits expressed in HEK cells have also been shown to require the presence of an AKAP79 for phosphorylation by PKA (Johnson et al., 1994, Gao et al., 1997). Immunocomplexes isolated from rat heart and brain extracts have also identified the α_{1C} subunit as associating with the AKAP MAP2B (Rubin, 1994, Davare et al., 1999). Given these associations, it might be predicted that more AKAPs or related proteins involved in localizing kinases and phosphatases to specific sites of action exist at synaptic terminals.

The yeast two-hybrid system has been previously used successfully to identify peptides that disrupt the α_{1B}/β_3 subunit interaction, results that could lead to the identification of antagonists with therapeutic potential (Young et al., 1998). N-type calcium channels undergo G-protein mediated inhibition by binding to $G_{\beta\gamma}$ proteins (Herlitze et al., 1996) and the yeast two-hybrid system was used to identify the binding of $G_{\beta 1}$ and $G_{\beta 2}$ isoforms to the I-II domain of the α_{1B} subunit (Garcia et al., 1998). Finally, the yeast two-hybrid system was responsible for identifying the interaction of the C-terminus of human and rat α_{1B} subunits with the first PDZ domain of rat Mint1, which led to identifying the α_{1B}/CASK interaction, and some clues into cell adhesion and neurotransmission functions involving N-type calcium channels (Schneider et al., 1994). While these calcium channel protein-protein interactions have been identified and provide some insight into channel physiology (see **Table 5**), it is likely that other proteins play a role in channel regulation and searching for novel interactions will provide further understanding of channel properties.

The yeast two-hybrid system has several advantages over other methods of identifying protein-protein interactions. This system looks at *in vivo* associations and there are several mechanisms to reduce false interactions. This system is also known for sensitivity and its ability to detect interactions other than covalent bonds and targets novel interactions that standard co-immunoprecipitations would likely miss (Nishimune et al., 1996). There is recent interest in determining weak and transient interactions in large native complexes and the yeast two-hybrid system can resolve these weak positive interactions. Since first introduced in the early 1980s, this powerful technique has become highly favored and with advances in genomic sequencing, has been widely utilized to define signal transduction cascades, metabolic pathways and protein complexes in species from bacteria to humans (Legrain and Selig, 2000).

Table 5. Identified Calcium Channel Protein Interaction Sites.

The table lists known calcium channel protein interactions. Some interactions have been well characterized and the binding sites are known while others require further biochemical studies to resolve the specific amino acid residues involved. 1. Rettig et al., 1996, 2. Charvin et al., 1997, 3. Sheng et al., 1997, 4. Maximov et al., 1999, 5. Leveque et al., 1998, 6. Zamponi et al., 1997, 7. Qin et al., 1997, 8. Dolphin et al., 1999, 9. Page et al., 1998, 10. Furukawa et al., 1998, 11. Rubin, 1994, Gao et al., 1997, Davare et al., 1999, 12. Davare et al., 2000, 13. Gray et al., 1997, Gray et al., 1998, 14. Schiff et al., 2000, 15. Beguin et al., 2001.

INTERACTION	BINDING SITE
Rat α_{1B} (rbB)/Syntaxin-1A	α_{1B} (718-859 and 832-963), SXN (181-288)[1]
Human α_{1A} (hBI)/Syntaxin-1A	α_{1A} (722-1036), SXN (181-288)[1]
Rat α_{1B} (rbB)/SNAP-25	α_{1B} (718-963)[1]
Human α_{1A} (hBI)/SNAP-25	α_{1A} (722-1036)[1]
Rat α_{1A} (rbA)/SNAP-25	α_{1A} (724-981)[1]
Rat α_{1A} (rBI)/Synaptotagmin	α_{1A} (780-969)[2]
Rat α_{1B} (rbB)/SNAP-25	α_{1B} (718-859 and 832-963)[1]
Rat α_{1B}/Synaptotagmin 1	α_{1B} (718-963), SYT 1 (262-385)[3]
Human α_{1B}/Mint1	α_{1B} (2201-2339), Mint1(647-751)[4]
Human α_{1B}/CASK	α_{1B} (2021-2339), CASK (547-742)[4]
Rabbit α_{1A}/Csp	α_{1A} (BI-2 isoform 780-969)[5]
Rat $\alpha_{1A}/G_{\beta\gamma}$	α_{1A}(416-434)[6]
Rat $\alpha_{1B}/G_{\beta\gamma}$	α_{1B} (410-428)[6]
Rat $\alpha_{1E}/G_{\beta\gamma}$	α_{1E} (356-451 and 2036-2074)[7]
Rat $\alpha_{1B}/G_{\beta\gamma}$	α_{1B} (1-55)[8]
Rat $\alpha_{1E}/G_{\beta\gamma}$	α_{1E} (1-50)[9]
$\alpha_{1B}/G_o\alpha$	α_{1B} (1931-1949)[10]
$\alpha_{1A}/G_o\alpha$	α_{1A}(2028-2046)[10]
Rat α_{1C}/AKAP MAP2B	association[11]
Rat α_{1C}/PP2	α_{1C}(1584-2140)[12]
Rabbit L-type/AKAP15	association[13]
Chick α_{1B}/RGS12	association[14]
Rat β_{1-3}/Kir/Gem	β_{1-3}(50-484)[15]

Putative cytoplasmic regions of the rat N-type α_{1B} subunit were chosen as regions to search for potential interactions for two main reasons. First, calcium channel intracellular domains, particularly the C-terminus, show a high degree of diversity among the different α_1 subunits, therefore protein interactions identified using these regions are likely to be unique to N-type channels. Secondly, several protein-protein interactions involving N-type channels had been previously characterized and could therefore act as positive controls in yeast two-hybrid screens.

Results

Yeast Two-hybrid Baits

Seven regions of the α_{1B} subunit were generated as Baits in order to search for protein-protein interactions in the yeast two-hybrid system (**Table 6, Figure 7**). Several of the Baits contained previously described protein interaction sites. For example, within Bait 7 the calcium channel β subunit binds to a conserved 18 amino acid sequence (QQ-E- -L-GY- -WI- - -E) in the intracellular loop between domains I and II of the α_1 subunit (Tanabe et al., 1987, Mikami et al., 1989, Mori et al., 1991, Williams et al., 1992a, Williams et al., 1992b, Pragnell et al., 1994). Also, within Bait 7, the $G_{\beta\gamma}$ subunits have been shown to bind the I-II linker of the α_{1B} subunit in the same region as the calcium channel β subunit (Zamponi et al., 1997). New data suggests other sites of interaction may exist which include the N- and C-terminus of the α_1 subunit (Tsien et al., 1988, Walker et al., 1998, Walker et al., 1999), regions within Bait 6 and Bait 4, respectively. For example, secondary binding sites for the $G_{\beta\gamma}$ subunit have been demonstrated in the N-terminus (Dolphin et al., 1999), a region within Bait 6 and the C-terminus, a region within Bait 4 (Qin et al., 1997).

The SNARE proteins syntaxin-1A, synaptotagmin and SNAP-25 bind to the Synprint site within

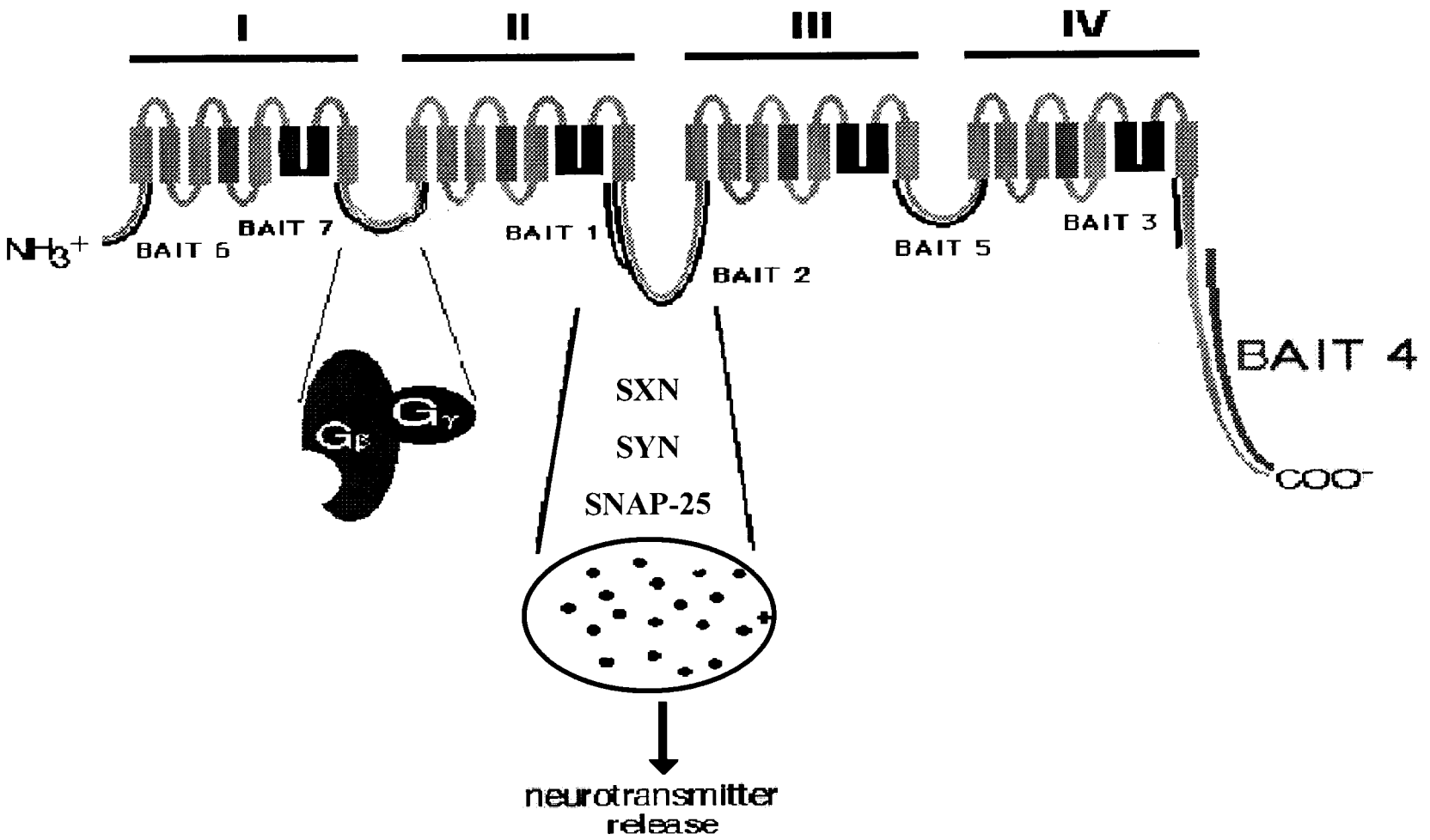
Table 6. Table of α_{1B} Subunit Bait Location.

Location and size of each region of the rbB-I α_{1B} subunit (accession M92905) used as a Bait in the yeast two-hybrid screen (Dubel et al., 1992). Oligonucleotides used for PCR amplification are described in the **Appendix (Figure 3)**.

α_{1B} Subunit Bait	α_{1B} Subunit Location	Number of Nucleotides	Number of Amino Acids	Oligonucleotides.
1	II-III linker (711 - 878 aa)	504	168	HG100, HG101
2	II-III linker (711 - 1148 aa)	1314	438	HG100, HG102
3	C-terminus (1728 - 1875 aa)	417	139	HG103, HG104
4	C-terminus (1972 - 2336 aa)	1086	362	HG105, HG106
5	III-IV linker (1419 - 1473 aa)	177	59	HG107, HG108
6	N-terminus (1 - 95 aa)	285	95	HG107A, HG108A
7	I-II linker (357 - 479 aa)	381	127	HG109, HG110

Figure 6. α_{1B} Subunit Bait Location.

Baits from the cytoplasmic regions of the α_{1B} subunit used in yeast two-hybrid system screening are labeled 1 to 7. Baits were amplified by PCR using oligonucleotides generated against the rat α_{1B} subunit sequence (rbB-I, accession M92905, Dubel et al., 1992). Bait 2 contains the Synprint site, the binding region of syntaxin-1A (SXN), synaptotagmin (SYN) and SNAP-25 (Leveque et al., 1992, Leveque et al., 1994, Sheng et al., 1994, Sheng et al., 1996). Bait 3 contains the EF hand, potential calcium binding region (Feng et al., 2001). Bait 4 and Bait 6 contain regions shown to bind the $G_{\beta/\gamma}$ subunit (Furukawa et al., 1998, Dolphin et al., 1999). Bait 7 includes the β subunit and a $G_{\beta/\gamma}$ binding site (Pragnell et al., 1994, Zamponi et al., 1997).


I
II
III
IV
NH_3^+
BAIT 6
BAIT 7
BAIT 1
BAIT 2
BAIT 5
BAIT 3
BAIT 4
COO^-
G_β
G_γ
SXN
SYN
SNAP-25
neurotransmitter
release

the II-III linker of the α_{1B} subunit (Leveque et al., 1992, Leveque et al., 1994, Sheng et al., 1994, Sheng et al., 1996) and is contained within Bait 1 and 2. The interaction of these proteins with the Synprint peptide *in vitro* showed calcium concentration-dependent binding with maximal binding at 20 μM calcium (Sheng et al., 1996). With this in mind, it was questionable whether binding with Bait 1 or 2 would demonstrate these interactions in the yeast nucleus where the calcium concentration may not be optimum.

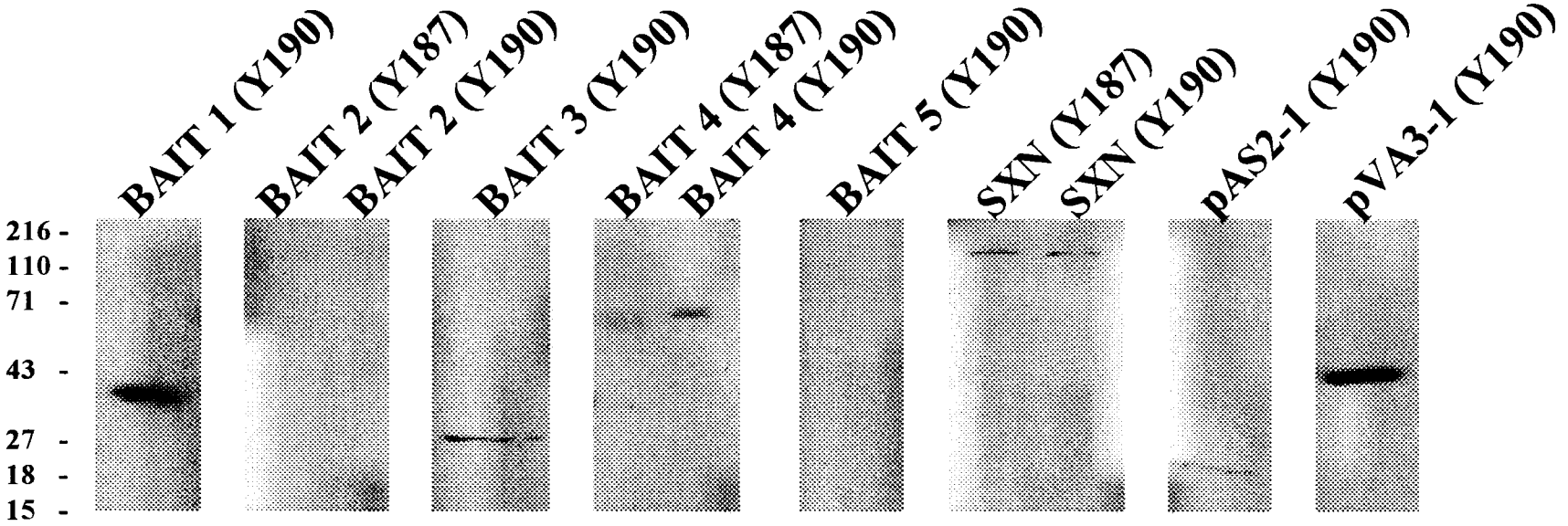
The Bait fusion protein primary sequences (**Appendix, Figure 4**) were examined for hydrophobic regions which could possibly attenuate targeting to the yeast nucleus (Silver et al., 1984, Chien et al., 1991). None of the Baits showed significant hydrophobicity suggesting that they would be acceptable for yeast two-hybrid analysis.

The PCR-amplified cDNA for each Bait region was cloned into the pAS2-1 vector and the DNA sequence confirmed. **Figure 7** shows that Baits 1, 3, 4 and syntaxin-1A were expressed in yeast. While Baits 1, 3 and 4 were the correct predicted size, syntaxin-1A appeared to have a higher molecular weight than predicted. Syntaxin demonstrated an interaction with itself in the mating assays. Baits 2 and 5 did not show expression in yeast. It is possible that these fusion proteins were toxic to yeast or that they aggregated such that nuclear targeting was disrupted. The usual method of detecting protein expression in yeast is by Western blot of protein extracted by whole cell lysis. A more informative method would be to use a nuclear extraction method which would reveal if the fusion proteins are being properly shuttled to the yeast nucleus.

Although no Bait 2 protein was detected by Western blot of extracted whole cell protein, yeast two-hybrid screening was still performed. The fact that no true positive interactions were detected confirms the likelihood that the Bait 2 peptide was not functioning properly in the yeast

Figure 7. Expression of Baits in Yeast.

Western blot of proteins expressed in yeast (Y190 or Y187 cell lines). Yeast cells transfected with a Bait (or control plasmid) were tested for Bait expression by whole cell protein extraction. Samples were run on 10% acrylamide gels and incubated with anti-Gal 4 binding domain antibody. Detection of Bait 2 and Bait 5 expression were not observed. Gal 4 binding domain (pAS2-1) and murine p53 (pVA3-1) were used as positive controls. Predicted MW; Bait 1 = 41 kDa, Bait 2 = 70 kDa, Bait 3 = 37 kDa, Bait 4 = 62 kDa, Bait 5 = 29 kDa, Syntaxin-1A (SXN) = 52 kDa, pAS2-1 = 22 kDa and pVA3-1 = 58 kDa. Syntaxin-1A expression showed a much higher molecular weight (approximately 100 kDa) likely due to formation of homodimers. Prestained high MW marker (BRL).


BAIT 1 (Y190)
BAIT 2 (Y187)
BAIT 2 (Y190)
BAIT 3 (Y190)
BAIT 4 (Y187)
BAIT 4 (Y190)
BAIT 5 (Y190)
SXN (Y187)
SXN (Y190)
pAS2-1 (Y190)
pVA3-1 (Y190)
216 -
110 -
71 -
43 -
27 -
18 -
15 -

and further screening with Baits 2 and 5 were not carried out.

The mating assay differs from the yeast two-hybrid screen in that the two candidate interacting proteins are isolated from one yeast strain and tested in another, eliminating false positives such as those proteins that activate reporter genes alone or activate reporter genes by interacting with the Gal4 binding domain (Bartel, 1993). The library protein was tested by itself (no additional plasmid transfected), the Gal4 binding domain (pAS2-1 plasmid alone), the α_{1B} C-terminus (pGAD10/Bait 4) or a negative control Bait (pGAD10/laminin plasmid). In the case of syntaxin-1A, reporter genes were activated when the yeast carried syntaxin-1A alone and not with the binding domain of Gal4 or laminin. This was verified by isolating the plasmids from the mated yeast, resulting in syntaxin-1A sequence and suggests that syntaxin-1A interacts with itself.

Yeast Two-hybrid Screening

Screening for protein-protein interactions using the yeast-two hybrid system consists of a series of preliminary steps including determining if Baits were toxic to yeast and if the Baits were capable of self activating reporter genes. Once the controls were confirmed and Bait expression in yeast was demonstrated, sequential or co-transformation methods of yeast transfection were tested for highest rate of efficiency. Although the sequential transformations gave higher density of colonies to screen and was an easier protocol to follow, the co-transformation method appeared more stringent in reducing background colonies and was preferred. Positive protein-protein interactions were first tested by the ability of a transfected yeast colony to survive on selective media that only supports growth when the presence of the Bait protein (pAS2-1/Gal4 binding domain plasmid) and the library protein (pGAD10/Gal4 activation domain plasmid) were present together. Those positive colonies were further tested in the β-galactosidase assay whereby expression of the LacZ reporter gene indicates a positive protein-protein interaction by

the restoration of the Gal4 promoter (binding domain and activation domain unite). Yeast colonies were then grown on selective media that allowed the library plasmid to be isolated from the Bait plasmid.

Two screens were carried out with Bait 1 by sequential transformation whereby $1x10^6$ colonies were examined for each screen and 16 potential candidate protein interactions were identified (**Table** 7). Once these interactions were further tested in the mating assay, reporter gene activity was lost for all 16 suggesting these candidates were not true interactions with Bait 1. Bait 2 was used in four separate yeast two-hybrid screens ranging from $2x10^4$ to $1x10^6$ independent colonies analyzed and resulted in two positive clones. These clones were found to be non-coding DNA fragments that falsely activated reporter genes and thus were not true protein-protein interactions for Bait 2. Bait 3 was used to screen $6.8x10^4$ and $8x10^6$ colonies resulting in 14 positive interactions. These positive clones were put through the mating assay and found to activate reporter genes without the presence of Bait 3 and were therefore eliminated as Bait 3 interactions.

Bait 4 contained the last 362 amino acids of the carboxyl terminus of the α_{1B} subunit. Two independent screens were performed of $2.4x10^5$ and $1.6x10^6$ independent clones and resulted in nine positive clones isolated. A search of GenBank by BLAST at NCBI (http://www.ncbi.nlm.nih.gov/BLAST/, Altschul et al., 1990) resulted in the identification of three different classes of proteins (see **Appendix Figure 6** for complete DNA and protein sequences). Seven of the clones encoded a Protein Kinase C Binding Protein Nell2 (U48245, Kuroda and Tanizawa, 1999), one clone encoded the LDL receptor related protein (NP_032538, Van Leuven et al., 1993), and one clone appeared to encode an IgG Fc binding protein (XM_016410, Harada et al., 1997) although the percentage similarity in the DNA sequences was

Table 7. Yeast Two-hybrid Screening Results.

Of the four Baits screened, only Bait 4 showed interactions with yeast clones that survived the rigorous selection process and mating assay and were considered true positive protein-protein interactions. (1) Method used in screening describes the technique of introducing DNA into yeast cells which can be carried out by sequential transformation (S) or co-transformation (C). Two yeast cell lines were used, Y190 and CG-1945. (2) After DNA was isolated out of the yeast colonies indicating a positive interaction in the yeast two-hybrid system, it was transformed into bacteria, amplified and isolated. The sequence was determined and run through GenBank to identify homology at the DNA level. Some DNA clones from the pGAD10 library were inserted in the 3'-5' orientation giving non-coding sequence (discarded) and others encode a poly T tail (poly T). (3) True positives were those clones that survived the mating assay, see Materials and Methods.

BAIT	SCREEN-ING METHOD[1]	YEAST COLONIES SCREENED	+ CLONES	SEQUENCE BLAST SEARCH RESULTS[2]	TRUE + [3]
1	S/Y190	$1.02x10^6$	4	- 3 X non-coding. - Maltose binding protein.	- -
1	S/Y190	$1X10^6$	12	- 6 X non-coding. - 1 X poly T. - Maltose binding protein. - Coatomer. - Rat Pancreatic Amylase. - Elastase. - Amyloid.	- - - - - - -
2	S/Y190	$1X10^6$	0	-	-
2	S/CG-1945	$1.7X10^4$	2	- 2 X non-coding.	-
2	C/Y190	$2.8X10^6$	0	-	-
2	S/Y190	$1X10^6$	0	-	-
3	S/Y190	$6.8X10^4$	0	-	-
3	C/Y190	$8X10^6$	14	- Not sequenced, lost in mating assay.	-
4	C/Y190	$2.4X10^5$	4	- 4 X PKC-BP Nell2 (U48245).	✔
4	S/Y190	$1.6X10^6$	5	- 3 X PKC-BP Nell2 (U48245). - Mouse LDL Receptor Related Protein 1 (NP_032538). - Human Fc fragment of IgG binding protein (XM_016410)	✔ ✔ -

low (less than 80 %). Syntaxin-1A was used in a mating assay to act as a positive control but did not show the expected binding to Bait 1. This may be due to the formation of syntaxin-1A homodimers (**Figure 7**) or relect the requirement for additional factors for efficient syntaxin-1A/Bait 1 interaction.

Cloning of Rat Nell2

Restriction enzyme analysis showed that the cDNA inserts in the seven yeast two-hybrid clones encoding Nell2 ranged from 1.5 to 2 kb (**Figure 8**). DNA sequence analysis showed that none of the clones contained full length coding sequence. In order to isolate the missing N-terminus portion, oligonucleotides were designed using the sequences isolated from the yeast library and the Nell2 sequence in GenBank. The full length Nell2 cDNA was then amplified by RT-PCR of whole rat brain RNA. The PCR product was subcloned into pGemTE and shown to contain a 2448 bp insert encoding an 816 aa protein (**Figure 9**). The estimated MW of Nell2 is 91069 Da (91 kDa) and the isoelectric point (pI) is 5.35 (http://ca.expasy.org/tools/pi_tool.html., Wilkins, 1998).

Comparison of the isolated Nell2 sequence to the previously published sequence (U48245, Kuroda and Tanizawa, 1999) showed ten nucleotide changes (**Appendix Figure 7**) resulting in seven amino acid substitutions (**Figure 10**). Two of the amino acids differed when the full length Nell2 was compared to the yeast two-hybrid clones isolated from screening with the α_{1B} subunit C-terminus (**Figure 11**). Both the previously published sequence and the yeast two-hybrid clones were generated from Clontech libraries by oligo(dT) and random primed PCR amplification and suggests the differences in cDNA sequences may be due to PCR errors.

Based on the overlapping yeast two-hybrid clones, a putative Nell2 binding region was

Figure 8. Agarose Gel of Positive Yeast two-hybrid Isolated cDNA.

Agarose gel showing results of EcoRI restriction digest on some yeast two-hybrid positive clones isolated from screening with Bait 4. Library inserts sizes ranged from 1500 to 2000 bp. pGAD10 vector = 6650 bp. Clones B4.15, B4.25, B4.66, B4.67, B4.69 and B4.84 are shown, B4.6 was also included in this group, but not shown here. This group was found to be homologous to the rat PKC-BP Nell2 protein (U48245, Kuroda et al., 1999).

MW (bp)
7000
6000
2000
1000
UNCUT 15
15
UNCUT 25
25
UNCUT 66
66
UNCUT 67
67
UNCUT 69
69
UNCUT 84
84

Figure 9. Complete cDNA and Protein Sequence of Rat Nell2.

Rat Nell2 was generated by RT-PCR using whole rat brain RNA. A 2448 bp coding region was amplified and the DNA sequence was determined manually (see **Appendix Figure 5** for complete oligonucleotide map). Rat Nell2 translates into a protein consisting of 816 amino acids as determined by Expasy's Translate Tool (http://www.expasy.org/tools/dna.html).

```
atggaatcccgggtattactgagaacgttctgcgtgatcctcgggctcgaagcggtttgg
 M  E  S  R  V  L  L  R  T  F  C  V  I  L  G  L  E  A  V  W 20
ggacttggtgtggacccctccctacagattgacgtcttatcagagttagaacttggggag
 G  L  G  V  D  P  S  L  Q  I  D  V  L  S  E  L  E  L  G  E 40
tccacagctggagtgcgccaagtctcaggactgcataatgggacgaaagccttcctcttc
 S  T  A  G  V  R  Q  V  S  G  L  H  N  G  T  K  A  F  L  F 60
caagattcccccagaagcataaaagcacccattgctacagctgagcggtttttccagaag
 Q  D  S  P  R  S  I  K  A  P  I  A  T  A  E  R  F  F  Q  K 80
ctgaggaataaacacgagttcacaattctggtgaccctgaaacagatccacttaaattcg
 L  R  N  K  H  E  F  T  I  L  V  T  L  K  Q  I  H  L  N  S 100
gaagtcattctctccatccaccacttggatcacaggtacctggaactggaaagcagcggc
 E  V  I  L  S  I  H  H  L  D  H  R  Y  L  E  L  E  S  S  G 120
caccggaatgagatcagactgcattaccgctctggaactcaccgcccgcacacggaagtg
 H  R  N  E  I  R  L  H  Y  R  S  G  T  H  R  P  H  T  E  V 140
tttccttatattttggctgatgccaagtggcacaagctctccttagccttcagtgcctcc
 F  P  Y  I  L  A  D  A  K  W  H  K  L  S  L  A  F  S  A  S 160
cacttaattttacacatcgactgcaacaagatctatgaacgagtggtggaaatgccttct
 H  L  I  L  H  I  D  C  N  K  I  Y  E  R  V  V  E  M  P  S 180
acagacttgcctctgggcaccacattttggttgggacagagaaataacgcacacgggtat
 T  D  L  P  L  G  T  T  F  W  L  G  Q  R  N  N  A  H  G  Y 200
tttaagggaataatgcaagatgtgcaattacttgtcatgccccaggggttcatcgctcag
 F  K  G  I  M  Q  D  V  Q  L  L  V  M  P  Q  G  F  I  A  Q 220
tgcccggatcttaatcgaacctgtccaacatgcaacgacttccatgggcttgtgcagaaa
 C  P  D  L  N  R  T  C  P  T  C  N  D  F  H  G  L  V  Q  K 240
atcatggagctgcaggacattttatcgaagacgtcagccaagttgtctagagctgaacaa
 I  M  E  L  Q  D  I  L  S  K  T  S  A  K  L  S  R  A  E  Q 260
cgaatgaacaggctggatcagtgctactgtgagcggacgtgcaccatgaagggagccacc
 R  M  N  R  L  D  Q  C  Y  C  E  R  T  C  T  M  K  G  A  T 280
taccgggagttcgagtcctggacagacggctgcaagaactgcacatgcttgaatgggacc
 Y  R  E  F  E  S  W  T  D  G  C  K  N  C  T  C  L  N  G  T 300
atccagtgcgagactctggtctgccctgctcccgactgcccggctaaatcggctccagcg
 I  Q  C  E  T  L  V  C  P  A  P  D  C  P  A  K  S  A  P  A 320
tacgtggatggcaagtgctgtaaggagtgcaagtccacctgccagttccaggggcggagc
 Y  V  D  G  K  C  C  K  E  C  K  S  T  C  Q  F  Q  G  R  S 340
tactttgagggagaaaggagcacagtcttctcagcttccggaatgtgcgtcttgtatgaa
 Y  F  E  G  E  R  S  T  V  F  S  A  S  G  M  C  V  L  Y  E 360
tgcaaggatcagaccatgaagcttgttgagaacgccggctgcccggctttagattgcccc
 C  K  D  Q  T  M  K  L  V  E  N  A  G  C  P  A  L  D  C  P 380
gagtctcatcagatcgccttgtctcacagctgctgcaaggtttgcaaaggttatgacttc
 E  S  H  Q  I  A  L  S  H  S  C  C  K  V  C  K  G  Y  D  F 400
tgttctgagaagcatacatgcacggagaactcagtctgcaggaacctgaacgacagggca
 C  S  E  K  H  T  C  T  E  N  S  V  C  R  N  L  N  D  R  A 420
gtgtgcagctgccgggatggtttccgggccctccgggaggacaatgcctactgtgaagac
 V  C  S  C  R  D  G  F  R  A  L  R  E  D  N  A  Y  C  E  D 440
attgacgagtgtgcagaggggcgccattactgccgtgagaacaccatgtgtgtgaacaca
 I  D  E  C  A  E  G  R  H  Y  C  R  E  N  T  M  C  V  N  T 460
ccgggctctttcctgtgtatctgccaaacagggtacatcagaatcgacgattactcgtgt
 P  G  S  F  L  C  I  C  Q  T  G  Y  I  R  I  D  D  Y  S  C 480
acggaacatgacgagtgcctcacaaaccagcacaactgtgacgagaacgctttgtgcttt
 T  E  H  D  E  C  L  T  N  Q  H  N  C  D  E  N  A  L  C  F 500
aacaccgttggaggtcacaactgcgtctgcaagcctggctacactgggaatggaaccacg
 N  T  V  G  G  H  N  C  V  C  K  P  G  Y  T  G  N  G  T  T 520
tgcaaagctttctgcaaagacggctgcagaaacgaaggtgcctgcattgctgccaatgtc
 C  K  A  F  C  K  D  G  C  R  N  E  G  A  C  I  A  A  N  V 540
tgtgcttgcccacaaggcttcaccggacccagctgtgagacagacattgatgagtgctct
 C  A  C  P  Q  G  F  T  G  P  S  C  E  T  D  I  D  E  C  S 560
gagggctttgttcagtgtgacagccgtgccaactgcattaacctgcctgggtggtaccac
 E  G  F  V  Q  C  D  S  R  A  N  C  I  N  L  P  G  W  Y  H 580
tgtgagtgcagagatggctaccatgacaatgggatgtttgcgccaggtggagaatcctgt
 C  E  C  R  D  G  Y  H  D  N  G  M  F  A  P  G  G  E  S  C 600
```

```
gaagatattgatgaatgtgggactgggaggcacagctgtgccaatgacaccatttgcttc
 E  D  I  D  E  C  G  T  G  R  H  S  C  A  N  D  T  I  C  F 620
aacttggacggtggctacgattgccggtgtccccatggaaagaactgcacaggggactgc
 N  L  D  G  G  Y  D  C  R  C  P  H  G  K  N  C  T  G  D  C 640
gtgcacgacgggaaagtcaaacacaacggccagatctgggtgctggagaacgacaggtgc
 V  H  D  G  K  V  K  H  N  G  Q  I  W  V  L  E  N  D  R  C 660
tctgtgtgttcctgccagactggatttgttatgtgtcgacggatggtctgtgactgcgaa
 S  V  C  S  C  Q  T  G  F  V  M  C  R  R  M  V  C  D  C  E 680
aaccccacagttgacctctcctgctgccctgagtgcgacccaaggctgagcagccagtgc
 N  P  T  V  D  L  S  C  C  P  E  C  D  P  R  L  S  S  Q  C 700
ctgcatcaaaacggggaaaccgtgtacaacagcggtgacacctgggtccaggattgccgt
 L  H  Q  N  G  E  T  V  Y  N  S  G  D  T  W  V  Q  D  C  R 720
cagtgccgctgcttgcaaggagaagttgactgctggcccctggcttgcccagaggtagag
 Q  C  R  C  L  Q  G  E  V  D  C  W  P  L  A  C  P  E  V  E 740
tgtgaatttagtgtccttcctgagaacgagtgctgcccacgctgtgtcaccgatccttgt
 C  E  F  S  V  L  P  E  N  E  C  C  P  R  C  V  T  D  P  C 760
caggctgacaccatccgcaatgacatcaccaaaacctgcctggacgagatgaacgtggtt
 Q  A  D  T  I  R  N  D  I  T  K  T  C  L  D  E  M  N  V  V 780
cgcttcactgggtcttcctggatcaagcacggcacggagtgcaccctctgccagtgcaag
 R  F  T  G  S  S  W  I  K  H  G  T  E  C  T  L  C  Q  C  K 800
aacggccacgtgtgctgctcagtggacccacagtgcctccaggagctgtga
 N  G  H  V  C  C  S  V  D  P  Q  C  L  Q  E  L  -        816
```

Figure 10. GenBank Rat Nell2 and Cloned Rat Nell2 Protein Sequence Alignment.

Protein sequence alignment of the rat Nell2 identified by S. Kuroda and submitted in GenBank (U48245, Kuroda et al., 1999) with the cloned Rat Nell2 generated by RT-PCR. The sequence alignment was done on ClustalX (Gap Opening 10, Gap Extension 0.20 and Scoring Matrix Gonnet 250, Thompson et al., 1997) with further analysis on GeneDoc (Nicholas, 1997). Ten nucleic acid differences (see **Appendix Figure** 7) result in seven amino acid changes between the two sequences.

```
                    *         20          *         40          *
Nell2   :  MESRVLLRTFCVILGLEAVWGLGVDPSLQIDVLSELELGESTAGVRQVSG  :   50
U48425  :  MESRVLLRTFCVILGLEAVWGLGVDPSLQIDVLSELELGESTAGVRQVPG  :   50
           MESRVLLRTFCVILGLEAVWGLGVDPSLQIDVLSELELGESTAGVRQV G

                   60          *         80          *         100
Nell2   :  LHNGTKAFLFQDSPRSIKAPIATAERFFQKLRNKHEFTILVTLKQIHLNS  : 100
U48425  :  LHNGTKAFLFQDSPRSIKAPIATAERFFQKLRNKHEFTILVTLKQIHLNS  : 100
           LHNGTKAFLFQDSPRSIKAPIATAERFFQKLRNKHEFTILVTLKQIHLNS

                    *         120         *         140         *
Nell2   :  EVILSIHHLDHRYLELESSGHRNEIRLHYRSGTHRPHTEVFPYILADAKW  : 150
U48425  :  GVILSIHHLDHRYLELESSGHRNEIRLHYRSGTHRPHTEVFPYILADAKW  : 150
            VILSIHHLDHRYLELESSGHRNEIRLHYRSGTHRPHTEVFPYILADAKW

                   160          *         180          *         200
Nell2   :  HKLSLAFSASHLILHIDCNKIYERVVEMPSTDLPLGTTFWLGQRNNAHGY  : 200
U48425  :  HKLSLAFSASHLILHIDCNKIYERVVEMPSTDLPLGTTFWLGQRNNAHGY  : 200
           HKLSLAFSASHLILHIDCNKIYERVVEMPSTDLPLGTTFWLGQRNNAHGY

                    *         220         *         240         *
Nell2   :  FKGIMQDVQLLVMPQGFIAQCPDLNRTCPTCNDFHGLVQKIMELQDILSK  : 250
U48425  :  FKGIMQDVQLLVMPQGFIAQCPDLNRTCPTCNDFHGLVQKIMELQDILSK  : 250
           FKGIMQDVQLLVMPQGFIAQCPDLNRTCPTCNDFHGLVQKIMELQDILSK

                   260          *         280          *         300
Nell2   :  TSAKLSRAEQRMNRLDQCYCERTCTMKGATYREFESWTDGCKNCTCLNGT  : 300
U48425  :  TSAKLSRAEQRMNRLDQCYCERTCTMKGATYREFESWTDGCKNCTCLNGT  : 300
           TSAKLSRAEQRMNRLDQCYCERTCTMKGATYREFESWTDGCKNCTCLNGT

                    *         320         *         340         *
Nell2   :  IQCETLVCPAPDCPAKSAPAYVDGKCCKECKSTCQFQGRSYFEGERSTVF  : 350
U48425  :  IQCETLVCPAPDCPAKSAPAYVDGKCCKECKSTCQFQGRSYFEGERSTVF  : 350
           IQCETLVCPAPDCPAKSAPAYVDGKCCKECKSTCQFQGRSYFEGERSTVF

                   360          *         380          *         400
Nell2   :  SASGMCVLYECKDQTMKLVENAGCPALDCPESHQIALSHSCCKVCKGYDF  : 400
U48425  :  SASGMCVLYECKDQTMKLVENAGCPALDCPESHQIALSHSCCKVCKGYDF  : 400
           SASGMCVLYECKDQTMKLVENAGCPALDCPESHQIALSHSCCKVCKGYDF

                    *         420         *         440         *
Nell2   :  CSEKHTCTENSVCRNLNDRAVCSCRDGFRALREDNAYCEDIDECAEGRHY  : 450
U48425  :  CSEKHTCMENSVCRNLNDRAVCSCRDGFRALREDNAYCEDIDECAEGRHY  : 450
           CSEKHTC ENSVCRNLNDRAVCSCRDGFRALREDNAYCEDIDECAEGRHY

                   460          *         480          *         500
Nell2   :  CRENTMCVNTPGSFLCICQTGYIRIDDYSCTEHDECLTNQHNCDENALCF  : 500
U48425  :  CRENTMCVNTPGSFLCICQTGYIRIDDYSCTEHDECLTNQHNCDENALCF  : 500
           CRENTMCVNTPGSFLCICQTGYIRIDDYSCTEHDECLTNQHNCDENALCF
```

```
                  *         520         *         540         *
Nell2   :  NTVGGHNCVCKPGYTGNGTTCKAFCKDGCRNGGACIAANVCACPQGFTGP  : 550
U48425  :  NTVGGHNCVCKPGYTGNGTTCKAFCKDGCKNGGACIAANVCACPQGFTGP  : 550
           NTVGGHNCVCKPGYTGNGTTCKAFCKDGC4N GACIAANVCACPQGFTGP

                 560         *         580         *         600
Nell2   :  SCETDIDECSEGFVQCDSRANCINLPGWYHCECRDGYHDNGMFAPGGESC  : 600
U48425  :  SCETDIDECSEGFVQCDSRANCINLPGWYHCECRDGYHDNGMFAPGGESC  : 600
           SCETDIDECSEGFVQCDSRANCINLPGWYHCECRDGYHDNGMFAPGGESC

                  *         620         *         640         *
Nell2   :  EDIDECGTGRHSCANDTICFNLDGGYDCRCPHGKNCTGDCVHDGKVKHNG  : 650
U48425  :  EDIDECGTGRHSCANDTICFNLDGGYDCRCPHGKNCTGDCVHDGKVKHNG  : 650
           EDIDECGTGRHSCANDTICFNLDGGYDCRCPHGKNCTGDCVHDGKVKHNG

                 660         *         680         *         700
Nell2   :  QIWVLENDRCSVCSCQTGFVMCRRMVCDCENPTVDLSCCPECDPRLSSQC  : 700
U48425  :  QIWVLENDRCSVCSCQTGFVMCQRMVCDCENPTVDLSCCPECDPRLSSQC  : 700
           QIWVLENDRCSVCSCQTGFVMC RMVCDCENPTVDLSCCPECDPRLSSQC

                  *         720         *         740         *
Nell2   :  LHQNGETVYNSGDTWVQDCRQCRCLQGEVDCWPLACPEVECEFSVLPENE  : 750
U48425  :  LHQNGETVYNSGDTWAQDCRQCRCLQEEVDCWPLACPEVECEFSVLPENE  : 750
           LHQNGETVYNSGDTW QDCRQCRCLQ EVDCWPLACPEVECEFSVLPENE

                 760         *         780         *         800
Nell2   :  CCPRCVTDPCQADTIRNDITKTCLDEMNVVRFTGSSWIKHGTECTLCQCK  : 800
U48425  :  CCPRCVTDPCQADTIRNDITKTCLDEMNVVRFTGSSWIKHGTECTLCQCK  : 800
           CCPRCVTDPCQADTIRNDITKTCLDEMNVVRFTGSSWIKHGTECTLCQCK

                  *
Nell2   :  NGHVCCSVDPQCLQEL  : 816
U48425  :  NGHVCCSVDPQCLQEL  : 816
           NGHVCCSVDPQCLQEL
```

Figure 11. Positive YIIH Clone DNA Sequences Aligned to Nell2.

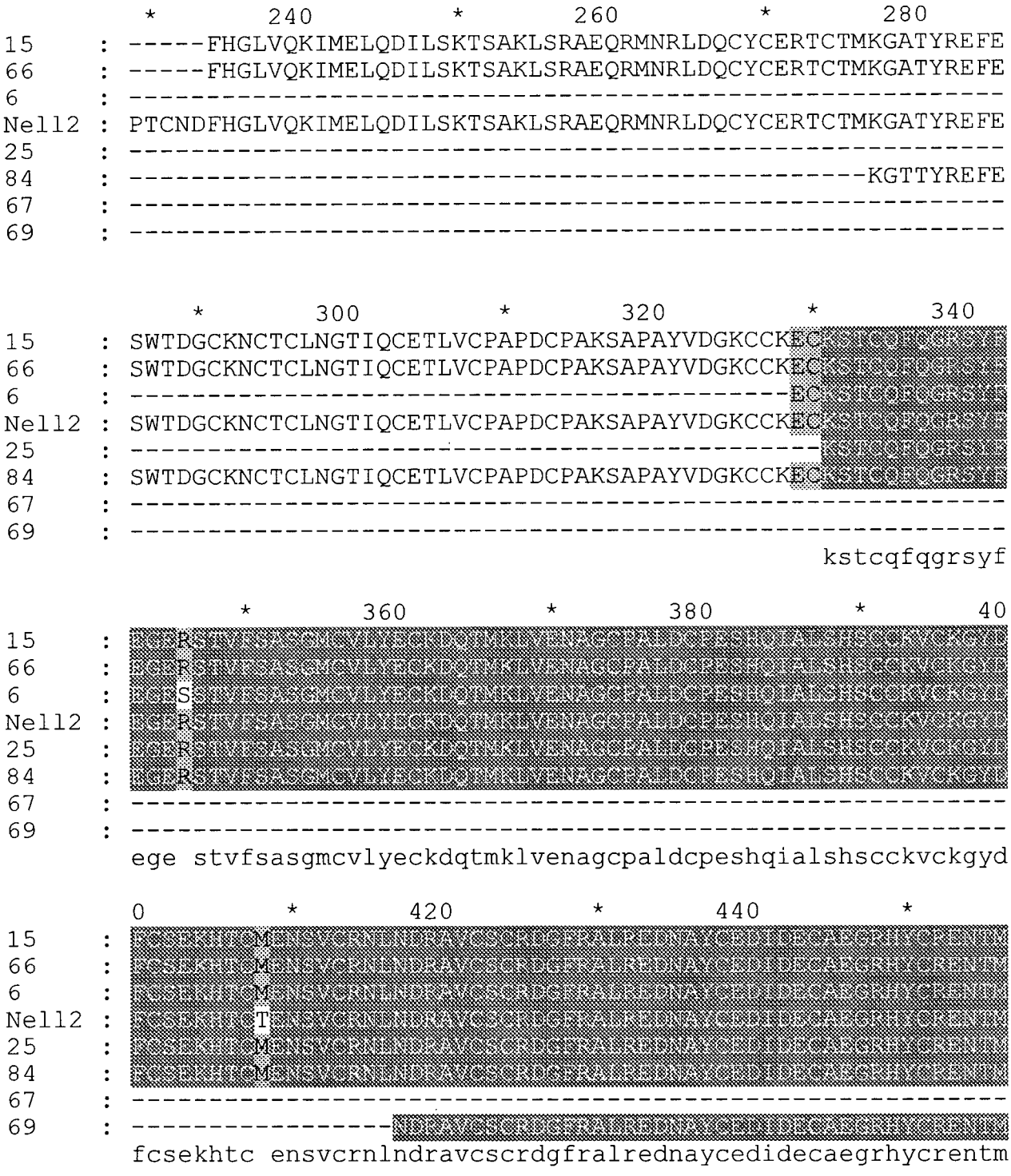
Yeast two-hybrid positive clones (B4.6, 15, 25, 66, 67, 69 and 84) were initially analyzed through BLAST at NCBI (http://www.ncbi.nlm.nih.gov/BLAST/) and found to be homologous to rat Nell2 (U48245, Kuroda et al., 1999). The amino acid sequences were then aligned to RT-PCR generated rat Nell2 using (Gap Opening 10, Gap Extension 10 and Scoring Matrix Gonnet 250, Thompson et al., 1997) with further analysis on GeneDoc (Nicholas, 1997) and found to differ by two amino acids.

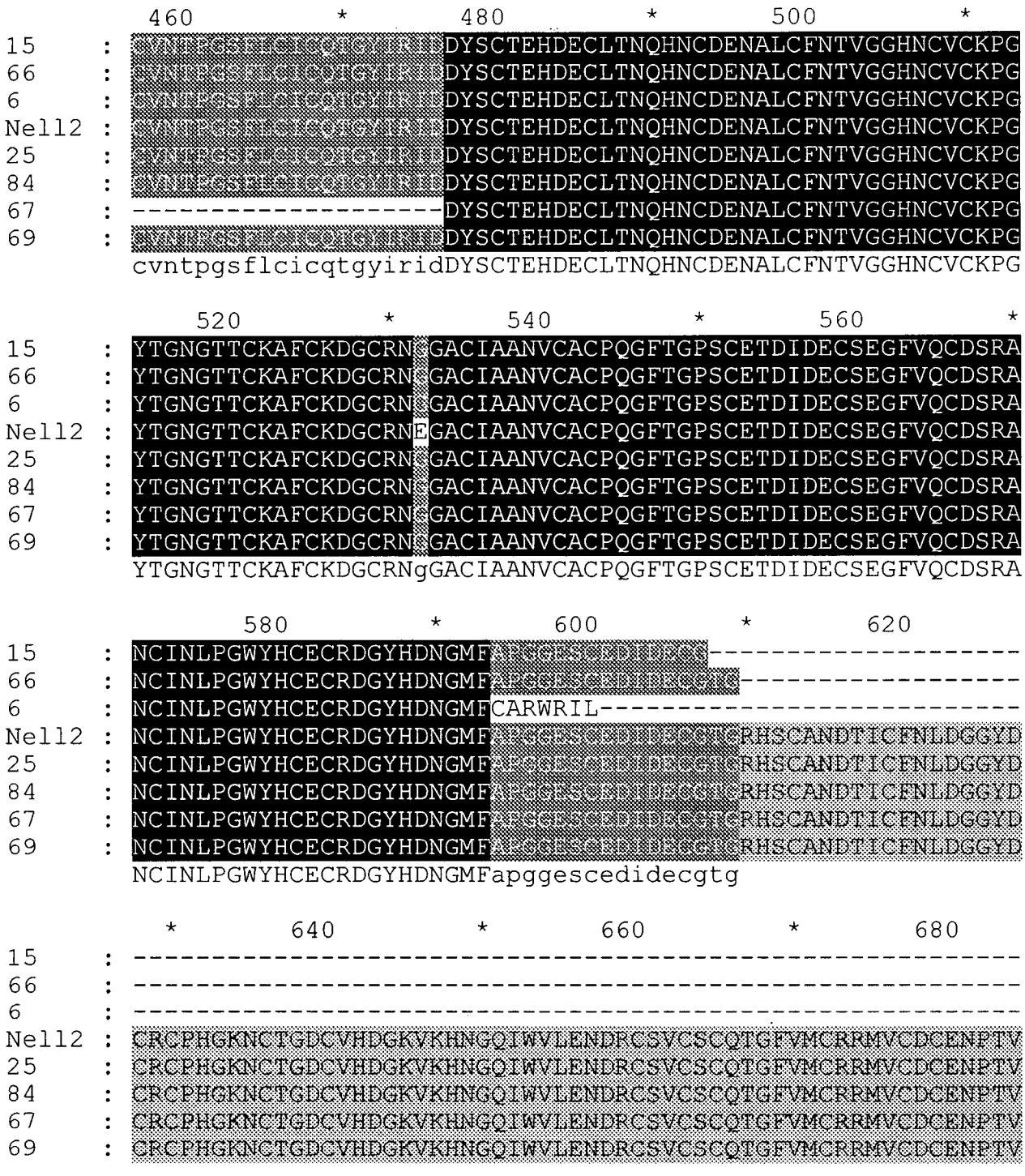
```
                 *         20         *         40         *
15     : ----------------------------------------------------------
66     : ----------------------------------------------------------
6      : ----------------------------------------------------------
Nell2  : MESRVLLRTFCVILGLEAVWGLGVDPSLQIDVLSELELGESTAGVRQVSGLHNGTKA
25     : ----------------------------------------------------------
84     : ----------------------------------------------------------
67     : ----------------------------------------------------------
69     : ----------------------------------------------------------

           60         *         80         *        100         *
15     : ----------------------------------------------------------
66     : ----------------------------------------------------------
6      : ----------------------------------------------------------
Nell2  : FLFQDSPRSIKAPIATAERFFQKLRNKHEFTILVTLKQIHLNSEVILSIHHLDHRYL
25     : ----------------------------------------------------------
84     : ----------------------------------------------------------
67     : ----------------------------------------------------------
69     : ----------------------------------------------------------

             120         *        140         *        160         *
15     : ----------------------------------------------------------
66     : ----------------------------------------------------------
6      : ----------------------------------------------------------
Nell2  : ELESSGHRNEIRLHYRSGTHRPHTEVFPYILADAKWHKLSLAFSASHLILHIDCNKI
25     : ----------------------------------------------------------
84     : ----------------------------------------------------------
67     : ----------------------------------------------------------
69     : ----------------------------------------------------------

                 180         *        200         *        220
15     : ----------------------------------------------------------
66     : ----------------------------------------------------------
6      : ----------------------------------------------------------
Nell2  : YERVVEMPSTDLPLGTTFWLGQRNNAHGYFKGIMQDVQLLVMPQGFIAQCPDLNRTC
25     : ----------------------------------------------------------
84     : ----------------------------------------------------------
67     : ----------------------------------------------------------
69     : ----------------------------------------------------------
```


240
260
280
15 : -----FHGLVQKIMELQDILSKTSAKLSRAEQRMNRLDQCYCERTCTMKGATYREFE
66 : -----FHGLVQKIMELQDILSKTSAKLSRAEQRMNRLDQCYCERTCTMKGATYREFE
6 : ----------
Nell2 : PTCNDFHGLVQKIMELQDILSKTSAKLSRAEQRMNRLDQCYCERTCTMKGATYREFE
25 : ----------
84 : ----------KGTTYREFE
67 : ----------
69 : ----------
300
320
340
15 : SWTDGCKNCTLNGTIQCETLVCPAPDCPAKSAPAYVDGKCCKEC
66 : SWTDGCKNCTLNGTIQCETLVCPAPDCPAKSAPAYVDGKCCKEC
6 : ----------EC
Nell2 : SWTDGCKNCTLNGTIQCETLVCPAPDCPAKSAPAYVDGKCCKEC
25 : ----------
84 : SWTDGCKNCTLNGTIQCETLVCPAPDCPAKSAPAYVDGKCCKEC
67 : ----------
69 : ----------
kstcqfqgrsyf
360
380
40
15 :
66 :
6 :
Nell2 :
25 :
84 :
67 : ----------
69 : ----------
ege stvfsasgmcvlyeckdqtmklvenagcpaldcpeshqialshscckvckgyd
420
440
15 :
66 :
6 :
Nell2 :
25 :
84 :
67 : ----------
69 : ----------
fcsekhtc ensvcrnlndravcscrdgfralrednaycedidecaegrhycrentm


460 * 480 * 500 *
15 : CVNTPGSFLCICQTGYIRIDDYSCTEHDECLTNQHNCDENALCFNTVGGHNCVCKPG
66 : CVNTPGSFLCICQTGYIRIDDYSCTEHDECLTNQHNCDENALCFNTVGGHNCVCKPG
6 : CVNTPGSFLCICQTGYIRIDDYSCTEHDECLTNQHNCDENALCFNTVGGHNCVCKPG
Nell2 : CVNTPGSFLCICQTGYIRIDDYSCTEHDECLTNQHNCDENALCFNTVGGHNCVCKPG
25 : CVNTPGSFLCICQTGYIRIDDYSCTEHDECLTNQHNCDENALCFNTVGGHNCVCKPG
84 : CVNTPGSFLCICQTGYIRIDDYSCTEHDECLTNQHNCDENALCFNTVGGHNCVCKPG
67 : ----------DYSCTEHDECLTNQHNCDENALCFNTVGGHNCVCKPG
69 : CVNTPGSFLCICQTGYIRIDDYSCTEHDECLTNQHNCDENALCFNTVGGHNCVCKPG
cvntpgsflcicqtgyiridDYSCTEHDECLTNQHNCDENALCFNTVGGHNCVCKPG
520 * 540 * 560 *
15 : YTGNGTTCKAFCKDGCRNGGACIAANVCACPQGFTGPSCETDIDECSEGFVQCDSRA
66 : YTGNGTTCKAFCKDGCRNGGACIAANVCACPQGFTGPSCETDIDECSEGFVQCDSRA
6 : YTGNGTTCKAFCKDGCRNGGACIAANVCACPQGFTGPSCETDIDECSEGFVQCDSRA
Nell2 : YTGNGTTCKAFCKDGCRNEGACIAANVCACPQGFTGPSCETDIDECSEGFVQCDSRA
25 : YTGNGTTCKAFCKDGCRNGGACIAANVCACPQGFTGPSCETDIDECSEGFVQCDSRA
84 : YTGNGTTCKAFCKDGCRNGGACIAANVCACPQGFTGPSCETDIDECSEGFVQCDSRA
67 : YTGNGTTCKAFCKDGCRNGGACIAANVCACPQGFTGPSCETDIDECSEGFVQCDSRA
69 : YTGNGTTCKAFCKDGCRNGGACIAANVCACPQGFTGPSCETDIDECSEGFVQCDSRA
YTGNGTTCKAFCKDGCRNgGACIAANVCACPQGFTGPSCETDIDECSEGFVQCDSRA
580 * 600 * 620
15 : NCINLPGWYHCECRDGYHDNGMF
66 : NCINLPGWYHCECRDGYHDNGMF
6 : NCINLPGWYHCECRDGYHDNGMFCARWRIL----------
Nell2 : NCINLPGWYHCECRDGYHDNGMF
25 : NCINLPGWYHCECRDGYHDNGMF
84 : NCINLPGWYHCECRDGYHDNGMF
67 : NCINLPGWYHCECRDGYHDNGMF
69 : NCINLPGWYHCECRDGYHDNGMF
NCINLPGWYHCECRDGYHDNGMFapggescedidecgtg
* 640 * 660 * 680
15 : ----------
66 : ----------
6 : ----------
Nell2 : CRCPHGKNCTGDCVHDGKVKHNGQIWVLENDRCSVCSCQTGFVMCRRMVCDCENPTV
25 : CRCPHGKNCTGDCVHDGKVKHNGQIWVLENDRCSVCSCQTGFVMCRRMVCDCENPTV
84 : CRCPHGKNCTGDCVHDGKVKHNGQIWVLENDRCSVCSCQTGFVMCRRMVCDCENPTV
67 : CRCPHGKNCTGDCVHDGKVKHNGQIWVLENDRCSVCSCQTGFVMCRRMVCDCENPTV
69 : CRCPHGKNCTGDCVHDGKVKHNGQIWVLENDRCSVCSCQTGFVMCRRMVCDCENPTV

```
                 *        700          *        720          *        740
15      :  ----------------------------------------------------------
66      :  ----------------------------------------------------------
6       :  ----------------------------------------------------------
Nell2   :  DLSCCPECDPRLSSQCLHQNGETVYNSGDTWVQDCRQCRCLQGEVDCWPLACPEVEC
25      :  DLSCCPECDPRLSSQCLHQNGETVYNSGDTWVQDCRQCRCLQGEVDCWPLACPEVEC
84      :  DLSCCPECDPRLSSQCLHQNGETVYNSGDTWVQDCRQCRCLQGEVDCWPLACPEVEC
67      :  DLSCCPECDPRLSSQCLHQNGETVYNSGDTWVQDCRQCRCLQGEVDCWPLACPEVEC
69      :  DLSCCPECDPRLSSQCLHQNGETVYNSGDTWVQDCRQCRCLQGEVDCWPLACPEVEC

                    *        760          *        780          *        8
15      :  ----------------------------------------------------------
66      :  ----------------------------------------------------------
6       :  ----------------------------------------------------------
Nell2   :  EFSVLPENECCPRCVTDPCQADTIRNDITKTCLDEMNVVRFTGSSWIKHGTECTLCQ
25      :  EFSVLPENECCPRCVTDPCQAD----------------------------------
84      :  EFSVLPENECCPRCVTDPCQADTIRNDITKTCLDEMNVVRFTGSSWIKHGTECTLCQ
67      :  EFSVLPENECCPRCVTDPCQADTIRNDITKTCLDEMNVVRFTGSSWIKHGTECTLCQ
69      :  EFSVLPENECCPRCVTDPCQADTIRNDITKTCLDEMNVVRFTGSSWIKHGTECTLCQ

            00          *
15      :  ------------------
66      :  ------------------
6       :  ------------------
Nell2   :  CKNGHVCCSVDPQCLQEL
25      :  ------------------
84      :  CKNGHVCCSVDPQCLQEL
67      :  CKNGHVCCSVDPQCLQEL
69      :  CKNGHVCCSVDPQCLQEL
```

determined for the α_{1B}/Nell2 interaction to be from 477 to 600 aa (**Figure 12**). Examination of the Nell2 protein sequence shows that it contains eleven potential casein kinase II phosphorylation sites, twelve potential PKC phosphorylation sites, two potential tyrosine phosphorylation sites, three aspartic acid and asparagine hydroxylation sites, twelve potential N-meristoylation sites, a VWFC domain, five EGF-like domains and a domain common to G-protein coupled receptors. The Nell2 sequence also includes a putative signal sequence and cleavage site (**Figure 13**). The EGF-like region of the Nell2 is found in the C-terminus (**Table 8**) and is within the α_{1B} putative binding region.

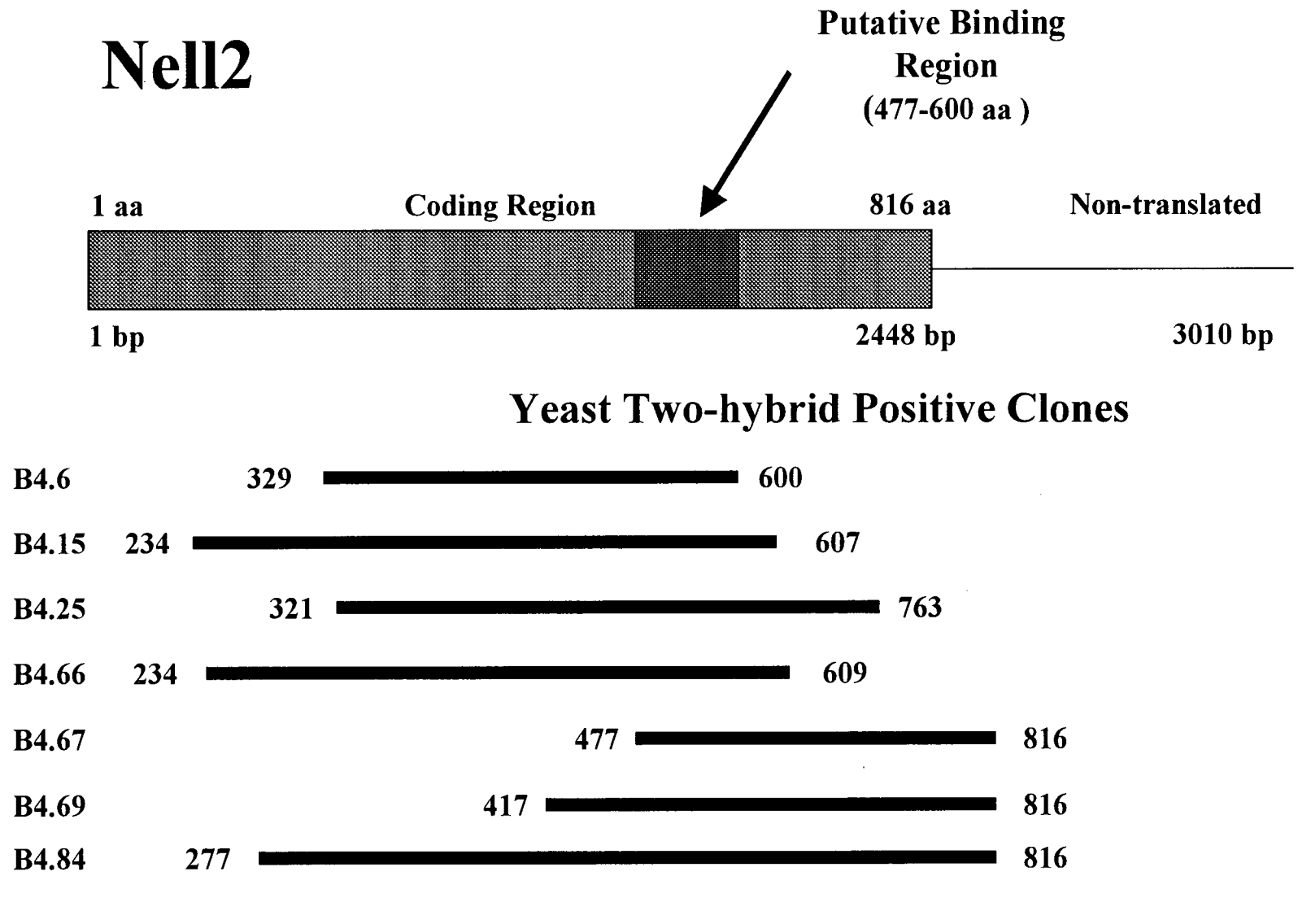
The Nell2 belongs to a family of proteins known as Nels, **N**euronal expressed proteins containing **E**GF-**L**ike repeats, (**Table 9).** Nel family sequences are highly conserved (see **Appendix Figure 8** for complete sequence) with two isoforms identified in chicken, human and rat. A third isoform or precursor protein has been identified in chicken and human. **Figure 14** shows a protein alignment of the Nel family members. The regions of highest sequence similiarity are the EGF-like repeats. Although the exact functions of the Nel family has not been identified, EGF-like repeats are genearlly important in protein-protein interactions (Carpenter and Zendegui, 1986; Davis, 1990; Rao et al., 1995).

LDL-receptor Related Protein

The C-terminus of the N-type calcium channel α_{1B} subunit (Bait 4) was also found to associate with a region of the low-density lipoprotein (LDL)-like receptor related protein 1 in the yeast two-hybrid system (NP_032538, Van Leuven et al., 1993). The yeast two-hybrid clone B4.46 complete amino acid sequence was found to be 99 % conserved to the region 2513 to 2734 aa (**Figure 15**). Within this region, there are three LDL receptor class A domains (**Figure 16**). LDLs are cholesterol-carrying lipoproteins of plasma. The LDL-receptor class A domains form

Figure 12. Putative Binding Region of Nell2/α_{1B}.

A putative binding region of Nell2 which binds to the C-terminus of the α_{1B} subunit (Bait 4) was determined by aligning the positive yeast two-hybrid clone DNA and cloned Nell2 DNA to find the region conserved amongst the yeast clones. Within the 816 amino acid Nell2 coding region, there was a 130 amino acid region is common to all positive yeast clones (1429 bp to 1822 bp, 477 aa to 607 aa).


Nell2
Putative Binding Region (477-600 aa)
1 aa
Coding Region
816 aa
Non-translated
1 bp
2448 bp
3010 bp
Yeast Two-hybrid Positive Clones
B4.6 329 600
B4.15 234 607
B4.25 321 763
B4.66 234 609
B4.67 477 816
B4.69 417 816
B4.84 277 816

the binding site for LDL (Yamamoto et al., 1984) and calcium (Van Driel et al., 1987) and are important for high-affinity binding of positively charged sequences (Mahley, 1988, Daly et al., 1995). Members of the LDL gene family include the low-density lipoprotein-receptor (LDL-R, LRP), megalin, very low-density lipoprotein-receptor (VLDL-R) and apolipoprotein E receptor 2 (apoER2) and were traditionally considered "recycling" cell surface receptors (Hussain, 2001). More recently, these proteins have been found to associate with adaptor and scaffold proteins and are now thought to play a role in cellular communication and signal transduction (Gotthardt et al., 2000).

Figure 13. Nell2 Protein Patterns.

The sequence of Rat Nell2 was analyzed for various consensus protein patterns and found to contain a signal sequence (http://HypothesisCreator.net/iPSORT/) with a signal peptide cleavage site (http://www.cbs.dtu.dk/services/SignalP/, scissors). Using ScanProsite (http://ca.expasy.org/tools/scnpsit1.html) several domains were found and some are labeled including 12 potential PKC phosphorylation sites (bolded and underlined), five EGF-like repeat domains (shown in **Impact** font, double underline indicates calcium binding EGF-like repeats, see **Table 8**), seven N-glycosylation sites, 13 casein kinase II phosphorylation sites, two tyrosine kinase phosphorylation sites, 12 N-myristoylation site, three aspartic acid and asparagine hydroxylation sites and two VWFC domain signatures. Nell2 sequence also contains two motifs common to G-protein coupled receptors (dashed underline). The putative α_{1B} subunit binding site shown outlined, includes one of the common G-protein coupled receptor motifs as well as two EGF-like repeats, one of which has calcium binding potential.

MESRVLLRTFCVILGLEAVWG✂LGVDPSLQIDVLSELELGESTAGVRQVSGLHNGTKAFLFQDS

PRSIKAPIATAERFFQKLRNKHEFTILVTLKQIHLNSEVILSIHHLDHRYLELESSGHRNEIRLH

YRSGTHRPHTEVFPYILADAKWHKLSLAFSASHLILHIDCNKIYERVVEMPSTDLPLGTTFWLGQ

RNNAHGYFKGIMQDVQLLVMPQGFIAQCPDLNRTCPTCNDFHGLVQKIMELQDILSKTSAKLSRA

EQRMNRLDQCYCERTCTMKGATYREFESWTDGCKNCTCLNGTIQCETLVCPAPDCPAKSAPAYVD

GKCCKECKSTCQFQGRSYFEGERSTVFSASGMCVLYECKDQTMKLVENAGCPALDCPESHQIALS

HSCCKVCKGYDFCSEKHTCTENSVCRNLNDRAVCSCRDGFRALREDNAYCEDIDECAEGRHYCRENT

MCVNTPGSFLCICQTGYIRIDDYSCTEHDECLTNQHNCDENALCFNTVGGHNCVCKPGYTGNGTTCKAFCK

DGCRNEGACIAANVCACPQGFTGPSCETDIDECSEGFVQCDSRANCINLPGWYHCECRDGYHDNGMFAPG

GESCEDIDECGTGRHSCANDTICFNLDGGYDCRCPHGKNCTGDCVHDGKVKHNGQIWVLENDRCSVCSC

QTGFVMCRRMVCDCENPTVDLSCCPECDPRLSSQCLHQNGETVYNSGDTWVQDCRQCRCLQGEVD

CWPLACPEVECEFSVLPENECCPRCVTDPCQADTIRNDITKTCLDEMNVVRFTGSSWIKHGTECT

LCQCKNGHVCCSVDPQCLQEL

Table 8. Nell2 EGF-like Domains.

Using ScanProsite (http://ca.expasy.org/tools/scnpsit1.html) the Nell2 amino acid sequence was analyzed and found to contain eight different EGF-like domains, three of which overlap. EGF-like repeats are known to be involved in cell proliferation, growth inhibition and differentiation (Handford et al., 1991, Rao et al., 1995). These regions are also thought to be important for protein-protein binding. These domains are characteristic of extracellular matrix proteins. (1 Expasy site, ScanProsite software at http://ca.expasy.org/tools/scnpsit1.html).

Type of EGF-like Domain[1]	Amino Acid Position
EGF-like domain signature 1.	541-552
EGF-like domain signature 2.	466-480 508-521 541-552 663-677
Calcium-binding EGF-like domain signature.	440-466 555-581 602-628

Table 9. The Nel Family of Proteins.

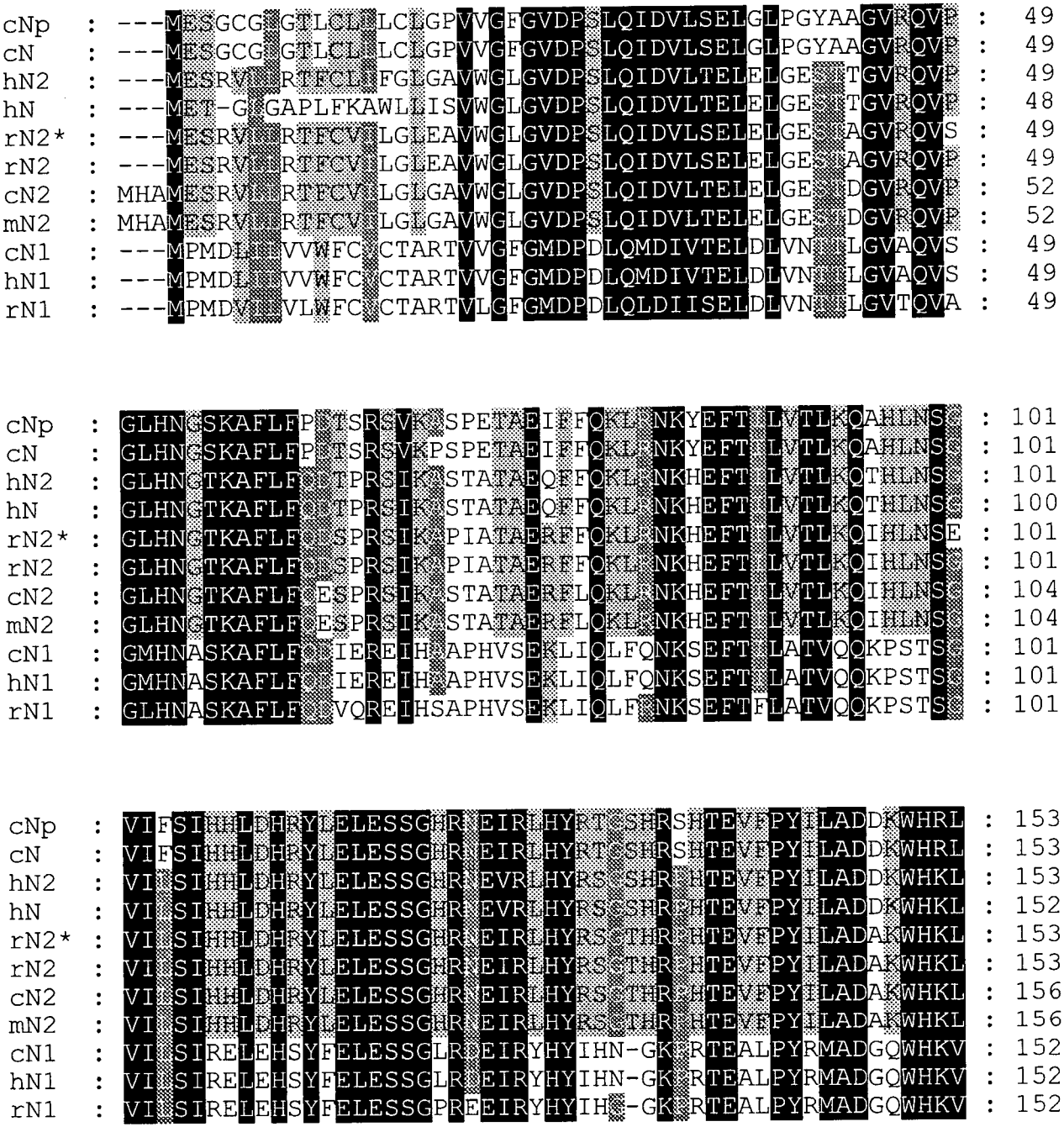
For Nel family member amino acid sequence abbreviations refer to **Figure 14** (and Nel family full length sequences, see **Appendix Figure 8**). Some family members are repeated as the same sequences have been submitted to GenBank under different names and accession numbers. Each clone was compared to the rat Nell2 sequence (rN2*) in ClustalX (Gap Opening 10, Gap Extension 0.20 and Scoring Matrix Gonnet 250, Thompson et al., 1997) and the alignment was statistically evaluated in GeneDoc (Nicholas, 1997) to give percent amino acid identity values. It appears that each species has at least two isoforms, Nell1 and Nell2, and a third isoform or precursor protein may also exist.

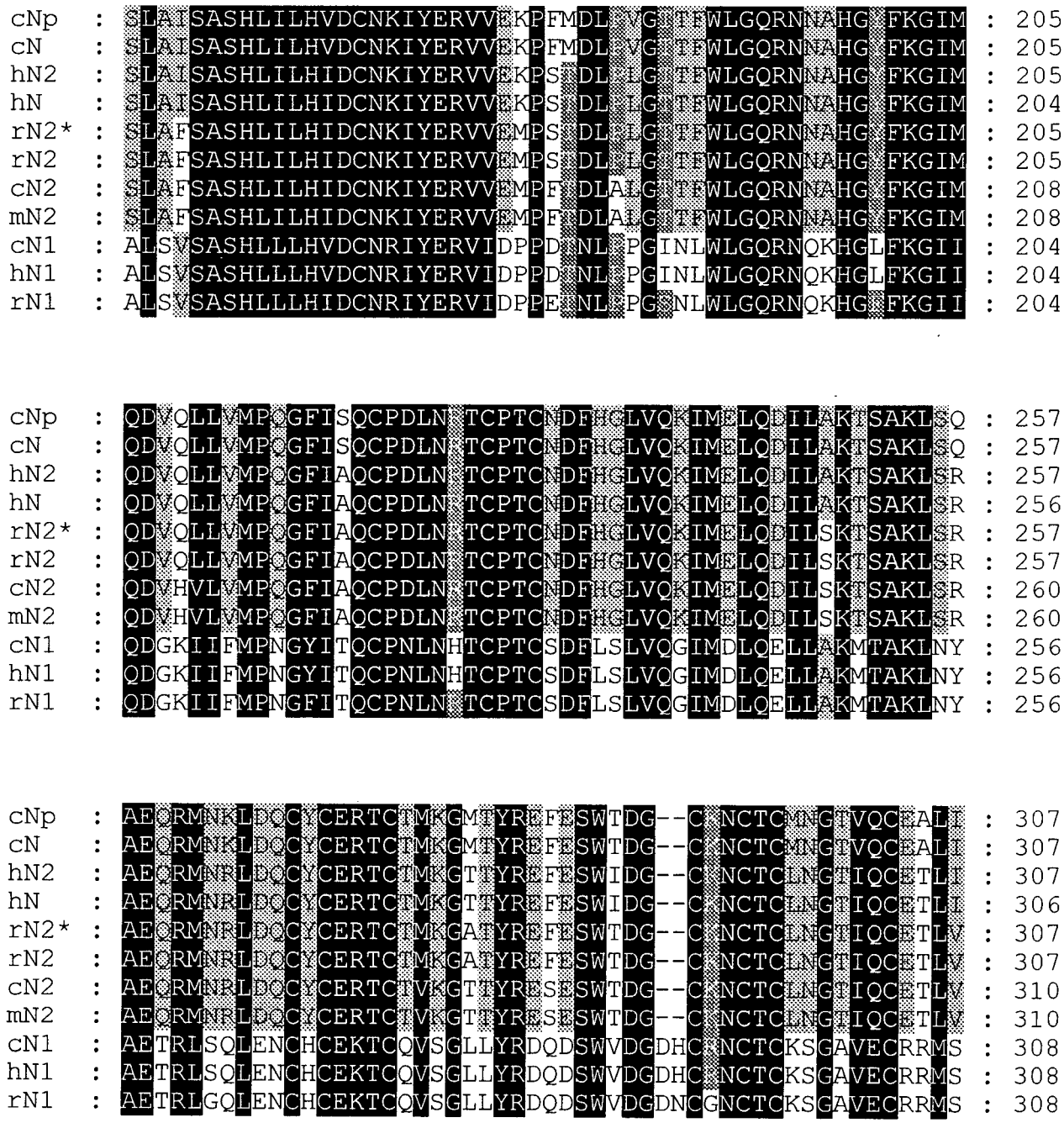
Sequence Abbreviation	Species/Clone	Accession Number	Number of Amino Acids	% Amino Acid Identity[2]	Reference
1 (rN2*)	Rat Cloned Nell2	N/A	816	N/A	Guthrie, 2001
2 (rN2)	Rat Protein Kinase C Binding Protein Nell2	NP_112332	816	97	Kuroda et al., 1999, Kuroda and Tanizawa, 1999
2 (rN2)	Rat Protein Kinase C Binding Protein Nell2	AAC72245	816	97	Kuroda et al., 1999, Kuroda and Tanizawa, 1999
2 (rN2)	Rat Protein Kinase C Binding Nell2 Protein Precursor	Q62918	816	97	Beckmann et al., 1998, Kuroda et al., 1999,
3 (cN2)	Chicken Nel-like 2 Homolog	NP_058023	819	93	-
4 (mN2)	Mouse Mel	AAB02924	819	93	-
4 (mN2)	Mouse Protein Kinase C Binding Protein Nell2 Precursor	Q61220	816	93	-
5 (hN2)	Human Nel-like 2	NP_006150	816	91	Watanabe et al., 1996, Kuroda et al., 1999, Luce and Burrows, 1999
5 (hN2)	Human Nel-related Protein 2	BAA11681	816	91	Watanabe et al., 1996
6 (hN)	Human Cerebral Protein-12	BAB46925	815	90	-
7 (cNp)	Chicken Nel Protein Precursor	Q90827	816	83	Matsuhashi et al., 1995, Beckmann et al., 1998
7 (cNp)	Chicken Mr 93 K	BAA13167	816	83	-

	Protein				
8 (cN)	Chicken Nel Protein	JP0076	835	76	Matsuhashi et al., 1995
9 (cN1)	Chicken Nel-like 1	NP_006148	810	53	Watanabe et al., 1996, Kuroda et al., 1999, Luce and Burrows, 1999
10 (rN1)	Rat Protein Kinase C Binding Protein Nell1	AAC72252	810	53	Kuroda et al., 1999, Kuroda and Tanizawa, 1999
10 (rN1)	Rat Nel-homolog Protein	T10756	810	53	-
10 (rN1)	Rat Protein Kinase C Binding Protein Nell1 Precursor	Q62919	810	53	Kuroda et al., 1999
10 (rN1)	Rat Protein Kinase C Binding Protein Nell1 Precursor	NP_112331	810	53	Kuroda et al., 1999, Kuroda and Tanizawa, 1999
11 (hN1)	Human Nel-related Protein	BAA11680	810	53	Watanabe et al., 1996
11 (hN1)	Human Protein Kinase C Binding Protein Nell1 Precursor	Q92832	810	53	Watanabe et al., 1996

Figure 14. Nel Family Sequence Alignment.

Nel family sequences (**Table 9**) were aligned in ClustalX (Gap Opening 10, Gap Extension 0.20 and Scoring Matrix Gonnet 250, Thompson et al., 1997) and further analyzed in GeneDoc (Nicholas, 1997). Amino acid sequences from rat, human, chicken and mouse were compared and found to be highly similar. The Nell2 putative binding region to the N-type α_{1B} subunit has been underlined and appears to be a homologous region among the family members. rN2* Rat Cloned Nell2, rN2 Rat Protein Kinase C Binding Protein Nell2 (NP_112332), cN2 Chicken Nel-like 2 Homolog (NP_058023), mN2 Mouse Mel (AAB02924), hN2 Human Nel-like 2 (NP_006150), hN Human Cerebral Protein-12 (BAB46925), cNp Chicken Nel Protein Precursor (Q90827), cN Chicken Nel Protein (JP0076), cN1 Chicken Nel-like 1 (NP_006148), rN1 Rat Protein Kinase C Binding Protein Nell1 (AAC72252), hN1 Human Nel-related Protein (BAA11680).

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hN2 : SLAISASHLILHIDCNKIYERVVEKPSTDLVLGTTEWLGQRNNAHGVFKGIM : 205
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hN1 : ALSVSASHLLLHVDCNRIYERVIDPPDTNLTPGINLWLGQRNQKHGLFKGII : 204
rN1 : ALSVSASHLLLHIDCNRIYERVIDPPETNLTPGSNLWLGQRNQKHGVFKGII : 204
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cN2 : QDVHVLVMPQGFIAQCPDLNTTCPTCNDFHGLVQKIMELQDILSKTSAKLSR : 260
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cN : AEQRMNKLDQCYCERTCTMKGMTYREFESWTDG--CNCTCMNGTVQCEALI : 307
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rN2 : AEQRMNRLDQCYCERTCTMKGATYREFESWTDG--CNCTCLNGTIQCETLV : 307
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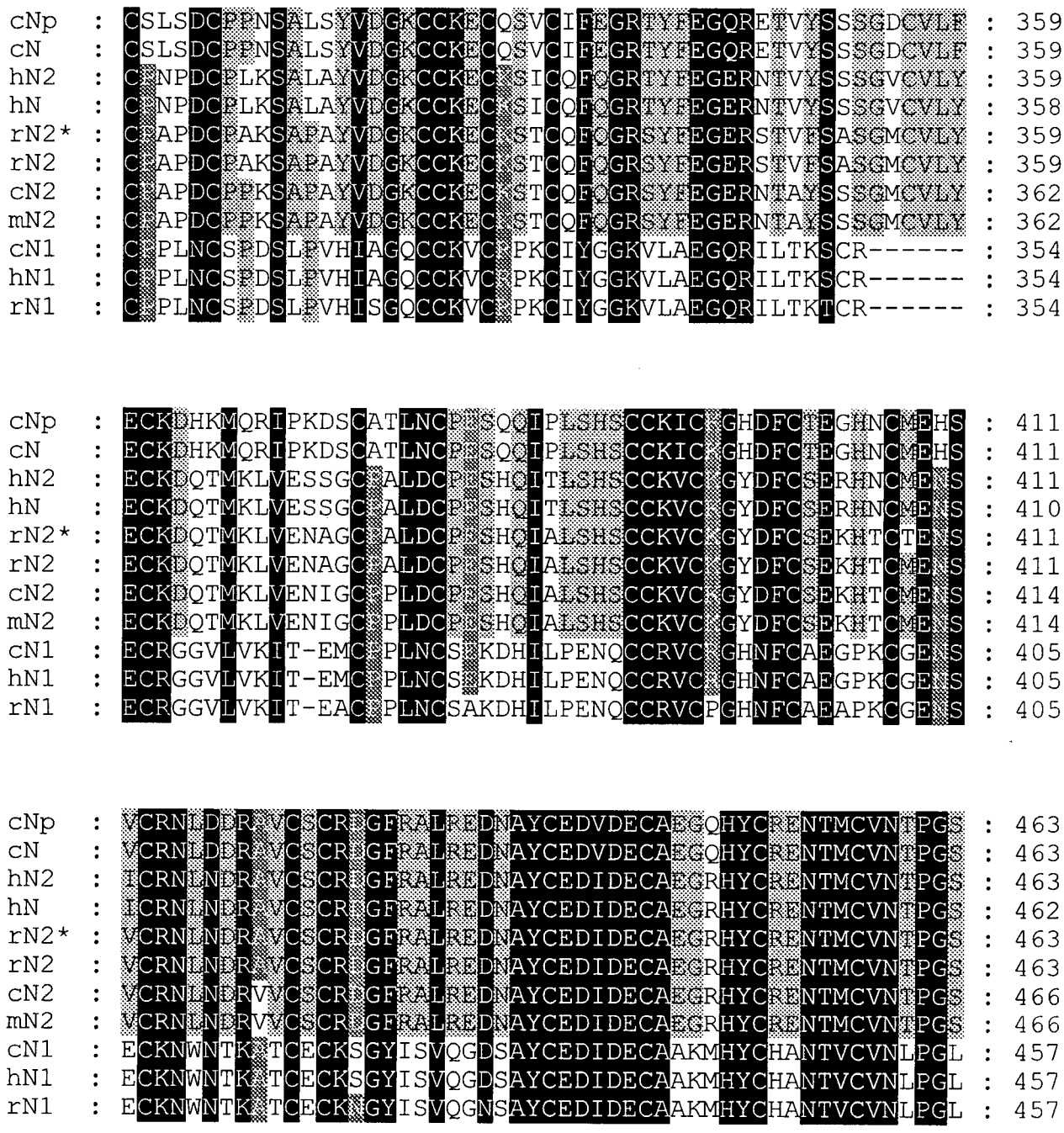
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hN : CRNPDCPLKSALAYVLGKCCKECRSICQFQGRTYREGERNTVYSSSGVCVLY : 358
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hN1 : CRPLNCSPDSLPVHIAGQCCKVCRPKCIYGGKVLAEGQRILTKSCR------ : 354
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cN : ECKDHKMQRIPKDSCATLNCPRSQQIPLSHSCCKICRGHDFCTEGHNCMEHS : 411
hN2 : ECKDQTMKLVESSGCRALDCPRSHQITLSHSCCKVCRGYDFCSERHNCMETS : 411
hN : ECKDQTMKLVESSGCRALDCPRSHQITLSHSCCKVCRGYDFCSERHNCMETS : 410
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mN2 : ECKDQTMKLVENIGCRPLDCPRSHQIALSHSCCKVCRGYDFCSEKHTCMETS : 414
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hN1 : ECRGGVLVKIT-EMCRPLNCSRKDHILPENQCCRVCRGHNFCAEGPKCGETS : 405
rN1 : ECRGGVLVKIT-EACRPLNCSAKDHILPENQCCRVCPGHNFCAEAPKCGETS : 405
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cN : VCRNLDERAVCSCRPGFRALREDNAYCEDVDECAEGQHYCRENTMCVNTPGS : 463
hN2 : ICRNLNERAVCSCRPGFRALREDNAYCEDIDECAEGRHYCRENTMCVNTPGS : 463
hN : ICRNLNERAVCSCRPGFRALREDNAYCEDIDECAEGRHYCRENTMCVNTPGS : 462
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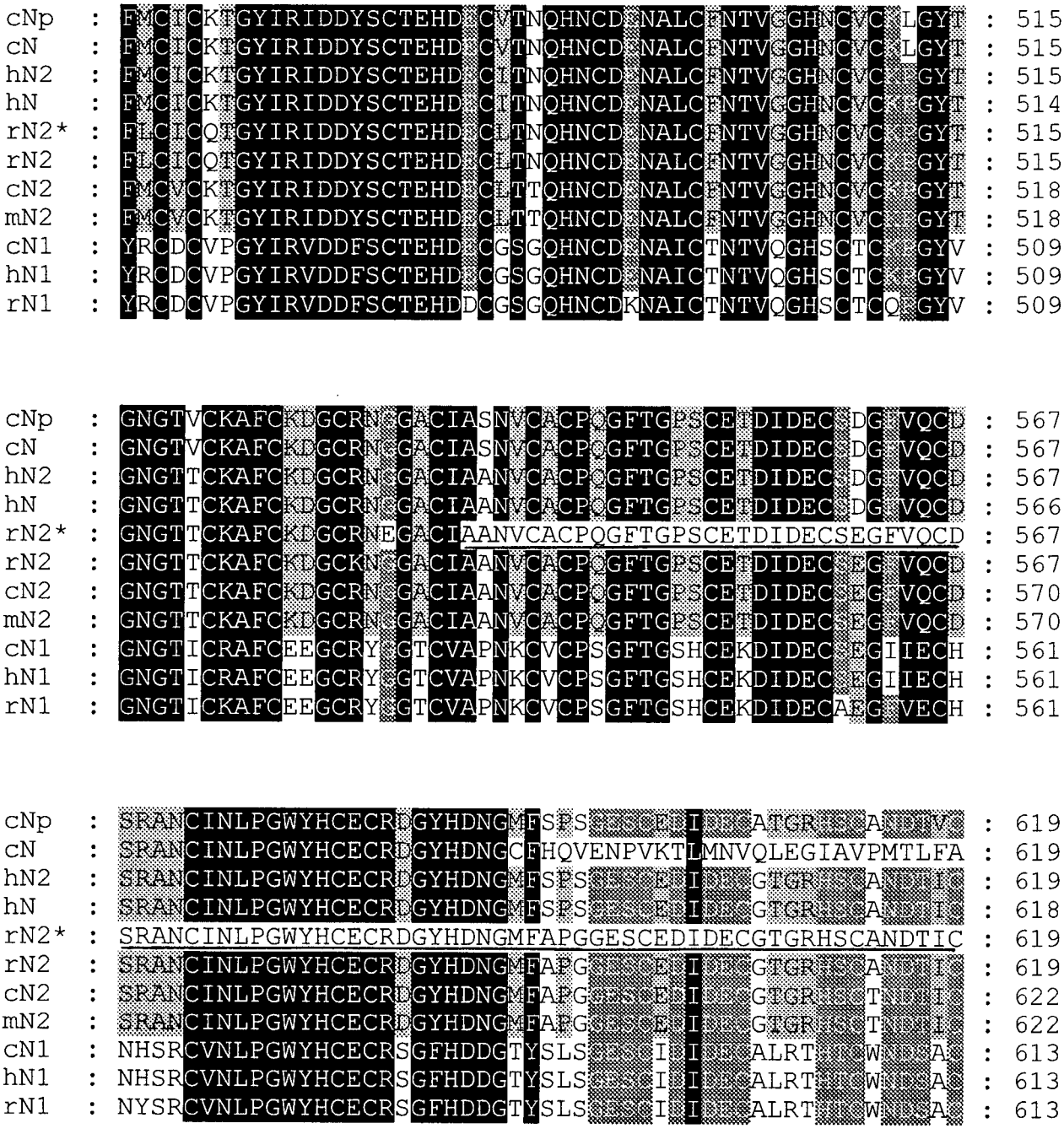

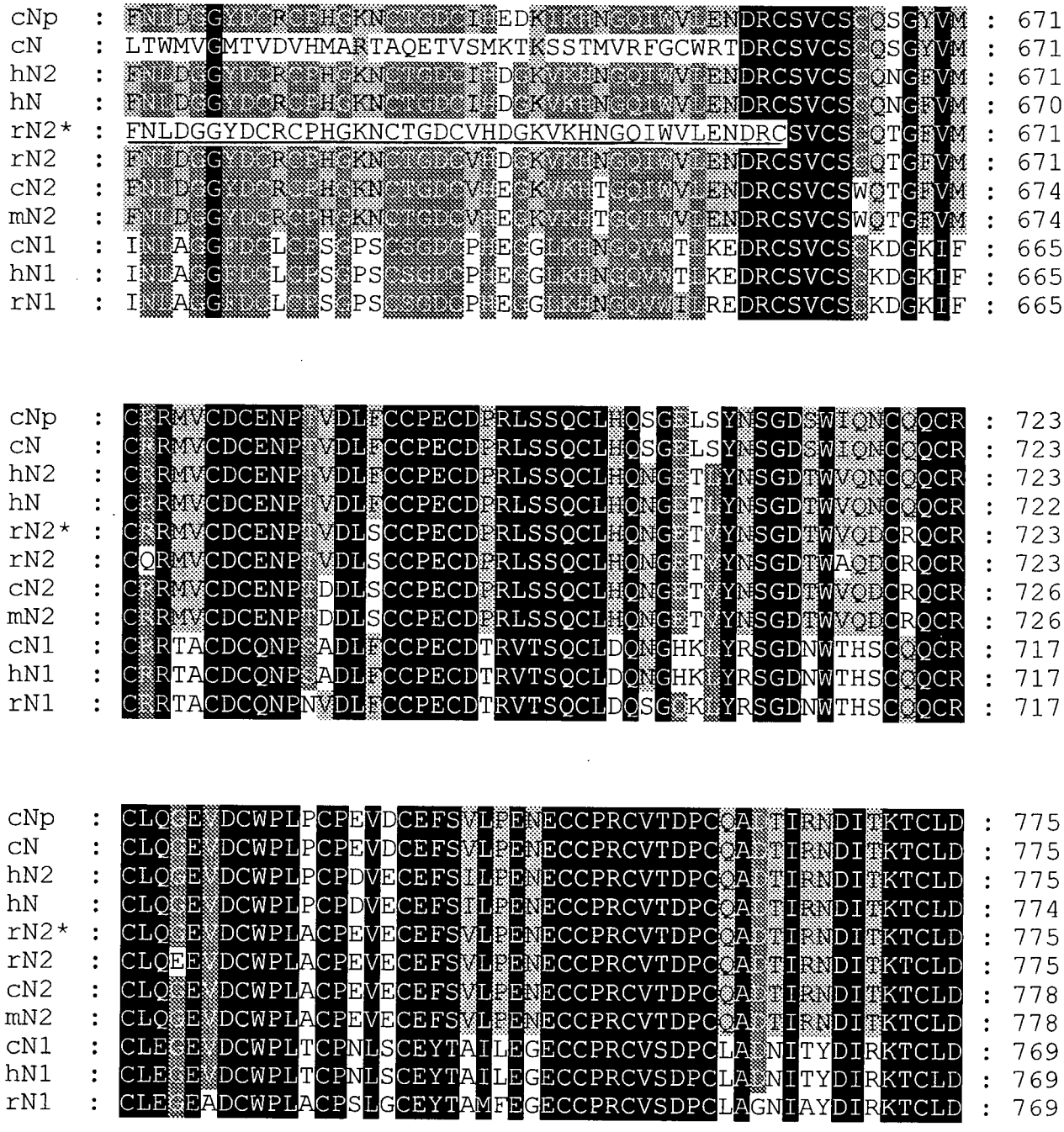
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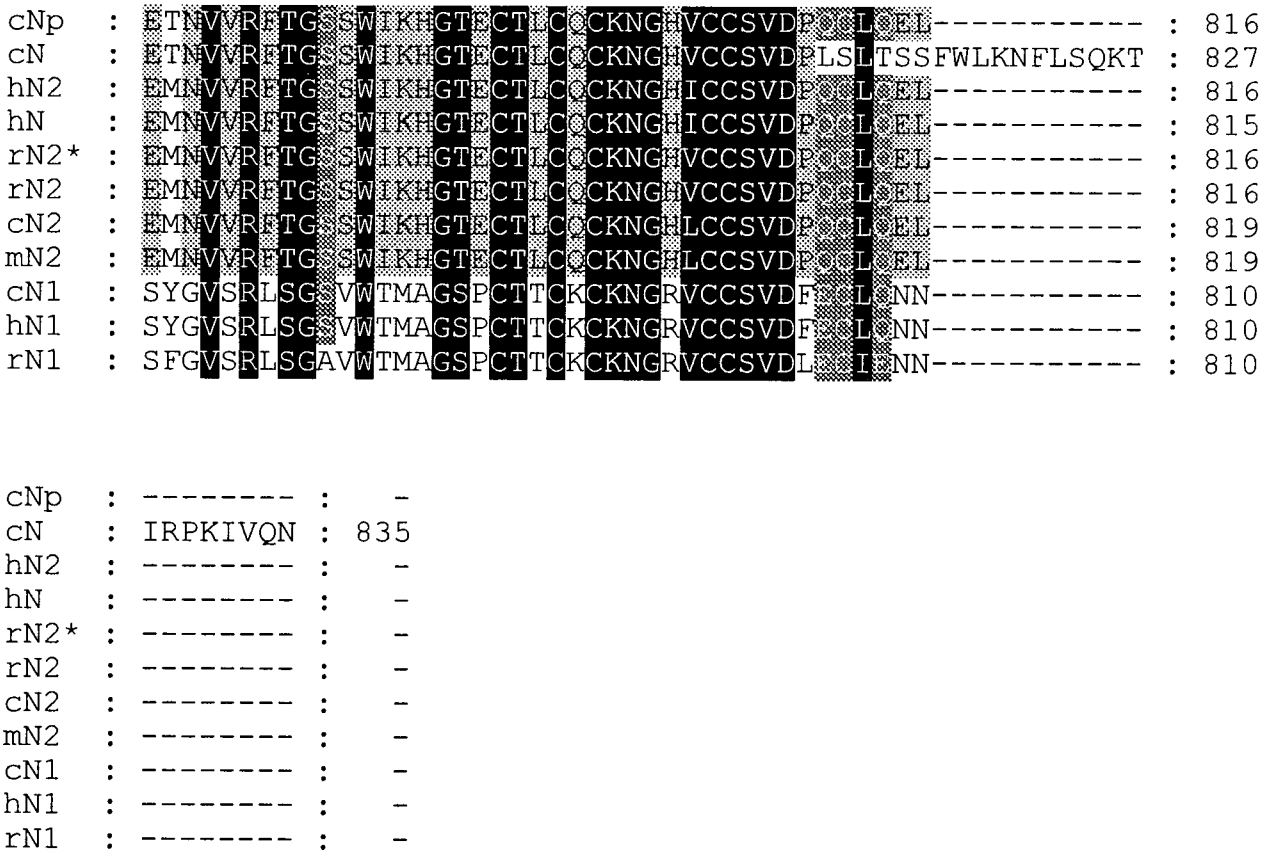
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cNp : -------- : -
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hN2 : -------- : -
hN : -------- : -
rN2* : -------- : -
rN2 : -------- : -
cN2 : -------- : -
mN2 : -------- : -
cN1 : -------- : -
hN1 : -------- : -
rN1 : -------- : -

Figure 15. LDL-receptor Related Protein Alignment to B4.46.

The yeast two-hybrid clone B4.46 exhibits 99% amino acid similarity to the mouse LDL Receptor Related Protein (LDL-like, 4545 aa, accession NP_032538, 3). Alignment was performed on ClustalX (Gap Opening 10, Gap Extension 0.20 and Scoring Matrix Gonnet 250, Thompson et al., 1997), and further analyzed on GeneDoc (Nicholas, 1997).

```
                2520          *       2540          *       2560
LDL-like :  ILQEDFTCRAVNSSCRAQDEFECANGECISFSLTCDGVSHCKDKSDEKPSYCNS
B4.46    :  ILQEDFTCRAMNSSCRAQDEFECANGECISFSLTCDGVSHCKDKSDEKPSYCNS
            ILQEDFTCRA6NSSCRAQDEFECANGECISFSLTCDGVSHCKDKSDEKPSYCNS

                *       2580          *       2600          *       2620
LDL-like :  RRCKKTFRQCNNGRCVSNMLWCNGVDYCGDGSDEIPCNKTACGVGEFRCRDGSC
B4.46    :  RRCKKTFRQCNNGRCVSNMLWCNGVD CGDGSDEIPCNKTACGVGEFRCRDGSC
            RRCKKTFRQCNNGRCVSNMLWCNGVD CGDGSDEIPCNKTACGVGEFRCRDGSC

                        *       2640          *       2660          *
LDL-like :  IGNSSRCNQFVDCEDASDEMNCSATDCSSYFRLGVKGVLFQPCERTSLCYAPSW
B4.46    :  IGNSSRCNQFVDCEDASDEMNCSATDCSSYFRLGVKGVLFQPCERTSLCYAPSW
            IGNSSRCNQFVDCEDASDEMNCSATDCSSYFRLGVKGVLFQPCERTSLCYAPSW

                  2680          *       2700          *       2720
LDL-like :  VCDGANDCGDYSDERDCPGVKRPRCPLNYFACPSGRCIPMSWTCDKEDDCENGE
B4.46    :  VCDGANDCGDYSDERDCPGVKRPRCPLNYFACPSGRCIPMSWTCDKEDDCENGE
            VCDGANDCGDYSDERDCPGVKRPRCPLNYFACPSGRCIPMSWTCDKEDDCENGE

                 *
LDL-like :  DETHCNKFC
B4.46    :  DETHCNKFC
            DETHCNKFC
```

Figure 16. LDL-receptor Related Protein Sequence Analysis.

LDL-receptor related protein amino acid sequence (2513 to 2734 aa) was searched for potential protein consensus sites using ScanProsite at Expasy (http://ca.expasy.org/tools/scnpsit1.html). Functional domains found included four N-glycosylation sites, six PKC phosphorylation sites (underlined), two Casein Kinase II phosphorylation sites, one tyrosine phosphorylation site (double underline), four N-myristoylation sites and three LDL receptor class A domains (bold).

ILQEDFTCRAVNSSCRAQDEFECANGE**CISFSLTCDGVSHCKDKSDEKPSYC**NSRRCK

KTFRQCNNGRCVSNMLWCNGVDYCGDGSDEIPCNKTACGVGEFRCRDGS**CIGNSSRC**

NQFVDCEDASDEMNCSATDCSSYFRLGVKGVLFQPCERTSLCYAPSWVCDGANDCGD

YSDERDCPGVKRPRCPLNYFACPSGR**CIPMSWTCDKEDDCENGEDETHC**NKFC

Chapter 4. Nell2 Cellular Localization

Background

Calcium channels have multiple functions in the nervous system and are differentially distributed at both the cellular and subcellular levels. While native N-type currents were originally thought to be exclusively expressed in neuronal tissues (Plummer et al., 1989, Plummer and Hess, 1991), some exceptions have been reported (Biagi and Enyeart, 1991). *In situ* immunohistochemistry using labeled ω-CgTx indicated native N-type channels to be widely distributed throughout CNS, including the cortex, hippocampus, olfactory bulb and cerebellum (Wagner et al., 1988, Bean, 1989, Jones et al., 1989 Takemura et al., 1989, Fortier et al., 1991, Catterall et al., 1993, Wheeler et al., 1994).

Staining of dorsal root ganglia (DRG) sensory neurons showed dendritic and axon terminal localization, and sympathetic neurons also showed growth cone staining (Lipscombe et al., 1988). In Purkinje cells, N-type channels localized at dendrite branch endings (Fortier et al., 1991) and clusters have been observed at synaptic terminals of hippocampal CA1 neurons (Jones et al., 1989). Cloned N-type calcium channel α_{1B} subunit expression patterns have confirmed the CNS localization patterns of the native channel. For example, Northern blot analyses show expression in the cerebral cortex, hippocampus, forebrain, midbrain, cerebellum and brainstem. Additionally, using a polyclonal antibody against α_{1B}, immunocytochemistry has revealed

staining on dendrites, presynaptic terminals and some cell bodies (Dubel et al., 1992, Williams et al., 1992, Fujita et al., 1993).

The Nell1 and Nell2 proteins were the first isolated from the chick (Matsuhashi et al., 1996) and mRNA was found highly expressed in all embryonic tissues and after hatching only in neuronal tissues (Matsuhashi et al., 1995, Matsuhashi et al., 1996). The human Nell1 and Nell2 mRNA has since been found to be highly expressed in adult brain with lower expression levels found in kidney (Watanabe et al., 1996). Human hemopoietic cell lines have been shown to express both Nell1 and Nell2 (Kuroda and Tanizawa, 1999). More recently using immunocytochemistry, rat Nell2 protein expression was found to be widely distributed within the brain, including the hippocampus, cerebral cortex, olfactory bulb, hypothalamus, thalamus, cerebellum and medulla (Luce and Burrows, 1999). No immunoreactivity was found in brain white matter or heart, lung, spleen, pancreas, kidney or testis, but was detected in the liver.

At the subcellular level, Nell2 staining is abundant in adult rat neurons in cell bodies and proximal dendrites, while no staining has been observed on peripheral dendrites or nerve fibers. Nell2 protein expression is high within the rough endoplasmic reticulum in neurons suggesting secretory packaging events. It has been hypothesized that Nell2 has several functions during development based on the different expression patterns found. In human and rat embryogenesis Nell2 is widely distributed in all tissues, but in the adult expression is specific to the nervous system suggesting Nell2 has a separate function in development and its contributions are still unknown (Oyasu et al., 2000).

Analysis of the Nell2 protein sequence shows a consensus signal sequence and cleavage site and suggests that Nell2 may be a secreted protein. Recombinant Nell2 protein has previously been

isolated from cytoplasm, membranes and extracellular fractions of transfected COS-7 cells (Kuroda and Tanizawa, 1999). In order to support the evidence of the potential interaction between Nell2 and the N-type calcium channel identified in the yeast two-hybrid system, the expression pattern of Nell2 was determined and compared to that of the α_{1B} N-type channel.

Results

Nell2 RNA Expression

RT-PCR was performed in order to determine the expression pattern of Nell2 in various tissues of adult male and female rats. Primer oligonucleotides were generated to the 3' non-coding region of Nell2 and amplified a unique 314 base pair DNA fragment. For comparison, oligonucleotides were also generated against the 3' coding region for the α_{1B} subunit. RT-PCR of Tubulin was used as a positive RNA control and was found in all samples tested. For each of the RT-PCR samples a Southern blot was probed with a $\gamma^{32}P$ labeled internal oligonucleotide to confirm the identification of the respective RT-PCR products. The Southern blot identified expression of Nell2 in brain stem, cerebellum, cortex and pituitary with trace amounts in eye and heart (**Figure 17**). No Nell2 expression was detected in adrenal gland, kidney, liver, ovary, seminal vesicle and spleen. RNA for the α_{1B} subunit was found to share expression of Nell2 in the brain stem, cerebellum, cortex, eye and pituitary. In contrast, α_{1B} mRNA was found in liver and kidney and only Nell2 was found in heart.

Since N-type channel/Nell2 binding experiments and electrophysiological recordings were carried out in HEK cells (see Chapter 5), the presence of endogenous Nell2 was also examined in HEK cells. Several attempts of RT-PCR were carried out using 3' non-translated

oligonucleotides to Nell2 but no DNA fragments were amplified. This may be due to species differences (human versus rat) in the 3' non-coding sequence. Human Nell was found to be weakly expressed in the kidney (Luce and Burrows, 1999) whereas rat Nell2 was not (Luce and Burrows, 1999, **Figure 17**). In other experiments RT-PCR was performed using oligonucleotides generated against the coding region of Nell2. Southern blotting confirmed the expression of Nell2 in both HEK 201 and HEK 108 cells, whereas the α_{1B} subunit was present only in the HEK cell line stably expressing N-type channels (HEK 108 cells; **Figure 18**).

Nell2 Protein Expression

In most species where Nell family members have been identified at least two homologs exist (Nell1 and Nell2), although only Nell2 has been found in mouse and a third precursor protein has been found in human and chick (Matsuhashi et al., 1995, Beckmann et al., 1998). The rat Nell1 and Nell2 isoforms are highly similar, sharing 53 % amino acid identity (**Table 9**). In order to generate a specific rat Nell2 antibody, the rat Nell1 amino acid sequence was compared to Nell2 and a unique region (from 314 to 388 aa) was selected for construction of a glutathione S-transferase (GST) fusion protein (**Figure 19**). The fusion protein was generated from E. coli (**Figure 20**) followed injection into rabbits with Freunds complete/incomplete adjuvant. The GST/Nell2 antigen was purified as two proteins with the lower band likely the result of degradation of the GST fusion protein (**Figure 20**). Purification of the antibody by CNBr affinity column showed loss of immunogenic response, possibly due to the relatively harsh elution conditions required for this protocol. Since crude sera gave a robust signal against the Nell2 antigen it was used in all experiments described below (**Figure 21**).

Two HEK cell lines, HEK 201 and HEK 108 were examined for Nell2 protein expression. The HEK 201 cells represent a variant of the native HEK 293 cell line with the addition of the simian

Figure 17. RT-PCR - RNA Expression of Tubulin, α_{1B} Subunit and Nell2 in Adult Rat Tissue.

Southern blot of RT-PCR products using oligonucleotides generated against rat tubulin (JM91 and JM92), the α_{1B} subunit (HG103 and HG105) and Nell2 (HG28 and HG29) for various tissues from adult male rat. The Southern blot was probed with a $\gamma^{32}P$ internal oligonucleotide to the region amplified (tubulin, JM93, α_{1B} subunit, R27 and Nell2, HG30). The 223 bp tubulin RT-PCR fragment was found in all RNA samples tested. RT-PCR products of the α_{1B} subunit were generated against the C-terminus (amino acids 1728 to 1875) using oligonucleotides which amplified a 443 base pair PCR product. RT-PCR products of Nell2 were generated using oligonucleotides against the 3' non-coding region resulting in a 314 bp PCR product. The identity of all of the RT-PCR products was confirmed by DNA sequencing.

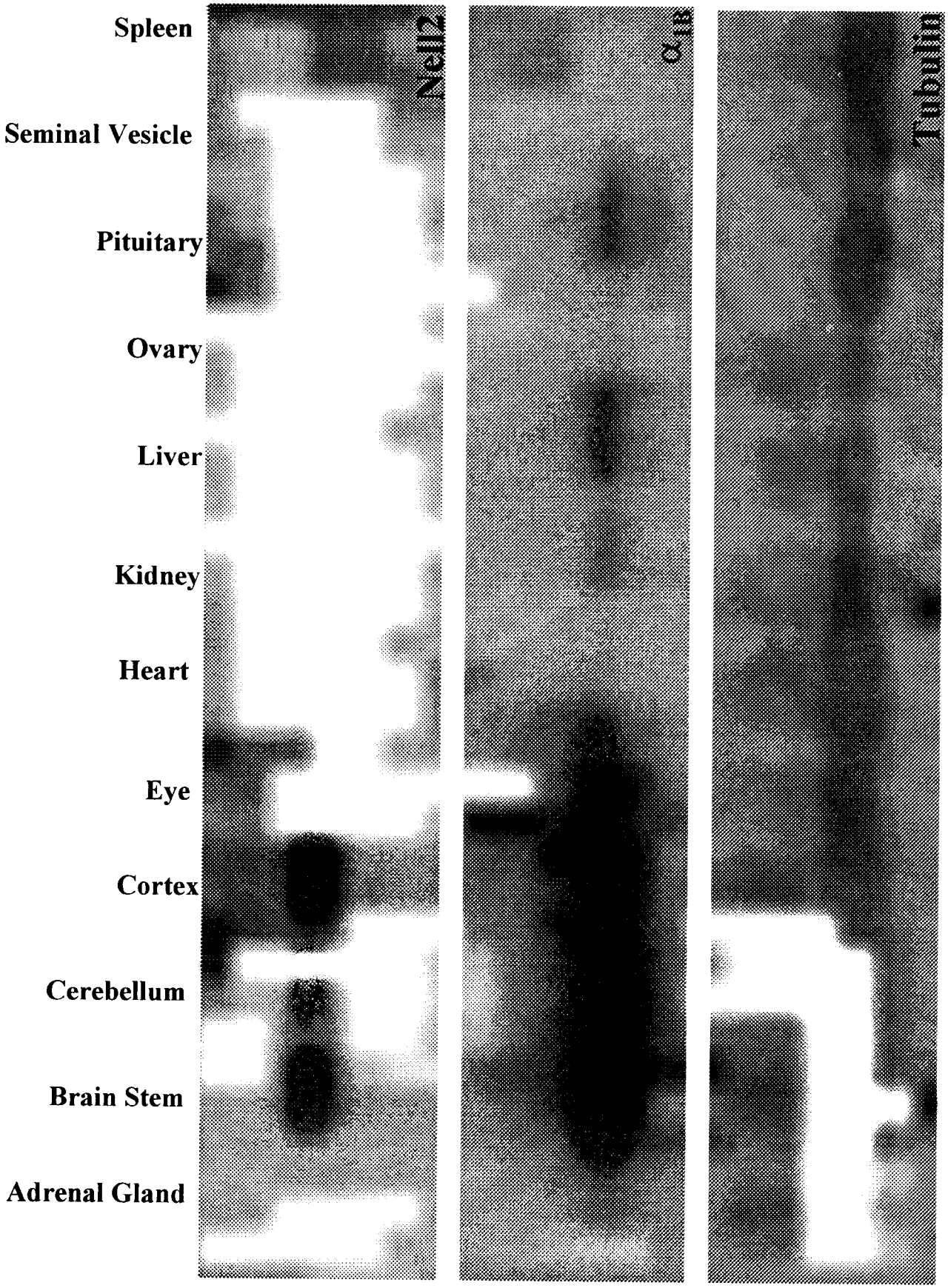

Spleen
Seminal Vesicle
Pituitary
Ovary
Liver
Kidney
Heart
Eye
Cortex
Cerebellum
Brain Stem
Adrenal Gland
Nell2
α1B
Tubulin

Figure 18. Expression of Tubulin, α_{1B} Subunit and Nell2 RNA in Human Cell Lines.

Southern blot of RT-PCR products using oligonucleotides generated against tubulin, the α_{1B} subunit and Nell2 using total RNA from Human Embryonic Kidney (HEK) cell lines. Tubulin and the α_{1B} subunit RT-PCR products were amplified, blotted and probed as described in Materials and Methods (**Figure 17**). The Nell2 oligonucleotides were generated against the coding region to amplify a 590 base pair fragment from HEK cell RNA. The HEK 108 cell line stably expresses the rat brain N-type calcium channel (α_{1B}, $\alpha_2\delta$ and β_{1b} subunits). The control for tubulin contained no template in RT-PCR reaction (negative control), whereas Nell2 and the α_{1B} subunit had the respective cDNA template in the PCR reaction (positive controls).

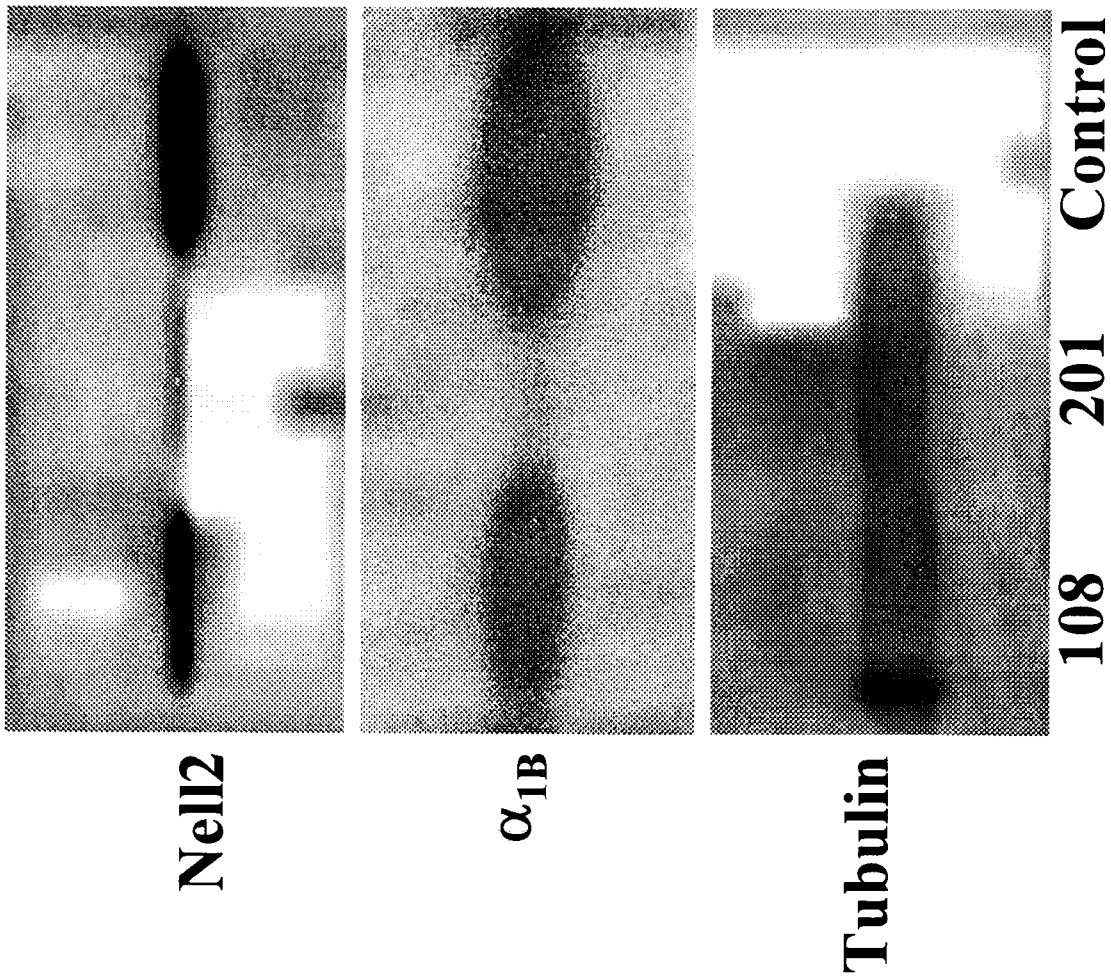
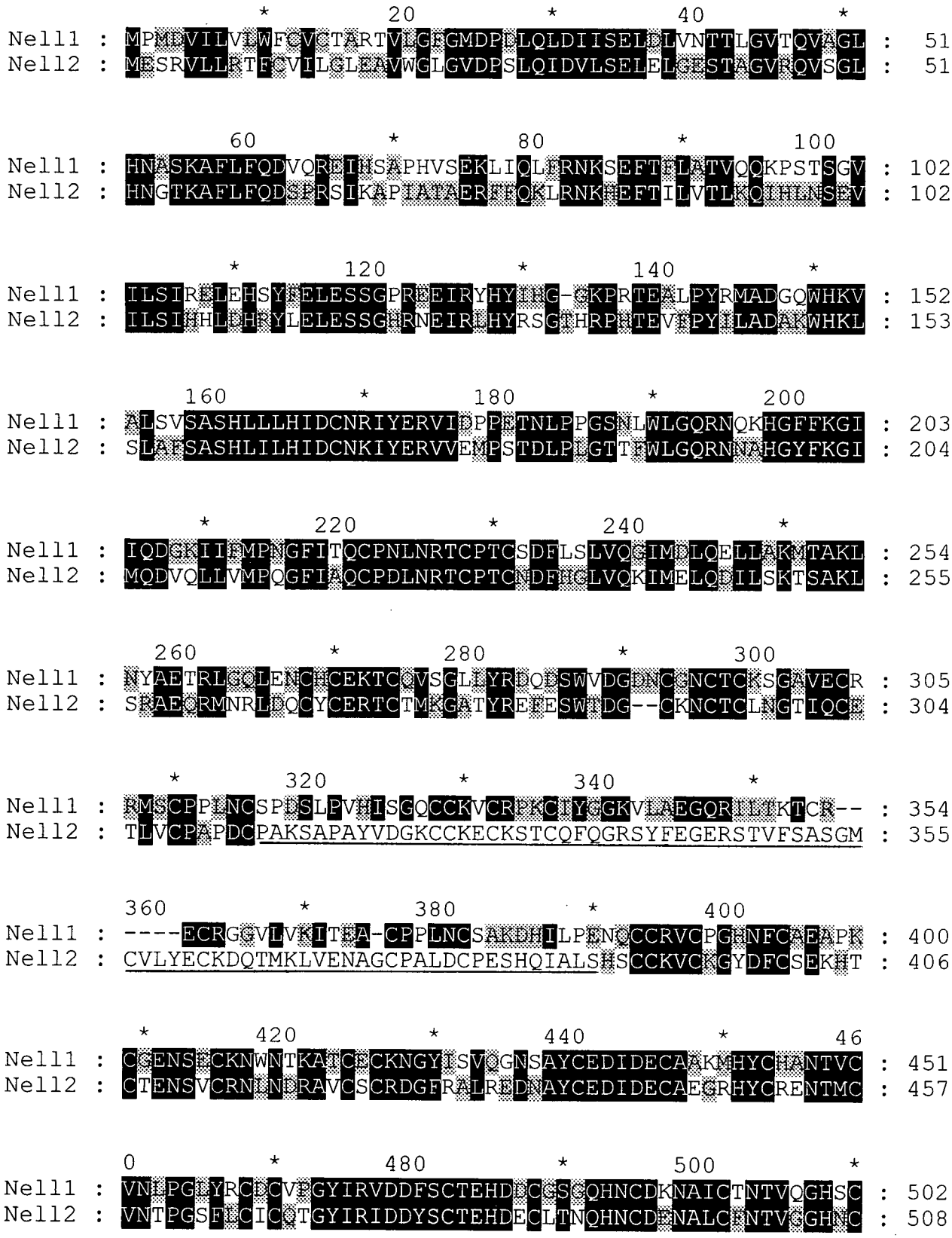

Nell2
α_{1B}
Tubulin
108
201
Control

Figure 19. Nell2 Antibody GST Fusion Protein.

Rat Nell1 and Nell2 were compared at the amino acid level to identify a region unique to Nell2. The amino acid sequence alignment was carried out using ClustalX (Gap Opening 10, Gap Extension 0.20 and Scoring Matrix Gonnet 250, Thompson et al., 1997) with further analysis on GeneDoc (Nicholas, 1997). A GST fusion protein was generated for Nell2 antibody production using amino acids 314 to 388 (underlined).


Nell1 : MPMLVILVLWFCVCTARTVLGFGMDPLLQLDIISELLLVNTTLGVTQVAGL : 51
Nell2 : MESRVLLRTFCVILGLEAVWGLGVDPSLQIDVLSELELCESTAGVRQVSGL : 51
Nell1 : HNASKAFLFQDVQREIHSAPHVSEKLIQLFRNKSEFTFLATVQQKPSTSGV : 102
Nell2 : HNGTKAFLFQDSPRSIKAPIATAERFFQKLRNKHEFTILVTLKQIHLNSEV : 102
Nell1 : ILSIREFEHSYFELESSGPREEIRYHYTHG-CKPRTEALPYRMADGQWHKV : 152
Nell2 : ILSIHHLLHRYLELESSGHRNEIRLHYRSGTHRPHTEVTPYILADAKWHKL : 153
Nell1 : ALSVSASHLLLHIDCNRIYERVIDPPFTNLPPGSNLWLGQRNQKHGFFKGI : 203
Nell2 : SLAFSASHLILHIDCNKIYERVVEMPSTDLPLGTTFWLGQRNNAHGYFKGI : 204
Nell1 : IQDGKIIFMPNGFITQCPNLNRTCPTCSDFLSLVQGIMDLQELLAKMTAKL : 254
Nell2 : MQDVQLLVMPQGFIAQCPDLNRTCPTCNDFHCLVQKIMELQDILSKTSAKL : 255
Nell1 : NYAETRLGQLENCFCEKTCQVSGLIYRDQLSWVDGDNCGNCTCKSGAVECR : 305
Nell2 : SRAECRMNRLDQCYCERTCTMKGATYRETESWTDG--CKNCTCLNGTIQCE : 304
Nell1 : RMSCPPLNCSPDSLPVHISGQCCKVCRPKCIYGGKVLAEGQRILTKTCR-- : 354
Nell2 : TLVCPAPDCPAKSAPAYVDGKCCKECKSTCQFQGRSYFEGERSTVFSASGM : 355
Nell1 : ----ECRGGVLVKITEA-CPPLNCSAKDHILPENQCCRVCPGENFCAEAPK : 400
Nell2 : CVLYECKDQTMKLVENAGCPALDCPESHQIALSHSCCKVCKGYDFCSEKHT : 406
Nell1 : CGENSECKNWNTKATCECKNGYISVQGNSAYCEDIDECAAKMHYCHANTVC : 451
Nell2 : CTENSVCRNLNDRAVCSCRDGFRALREDNAYCEDIDECAEGRHYCRENTMC : 457
Nell1 : VNLPGLYRCDCVPGYIRVDDFSCTEHDECCSGQHNCDKNAICTNTVQGHSC : 502
Nell2 : VNTPGSFLCICQTGYIRIDDYSCTEHDECLTNQHNCDENALCFNTVGGHNC : 508

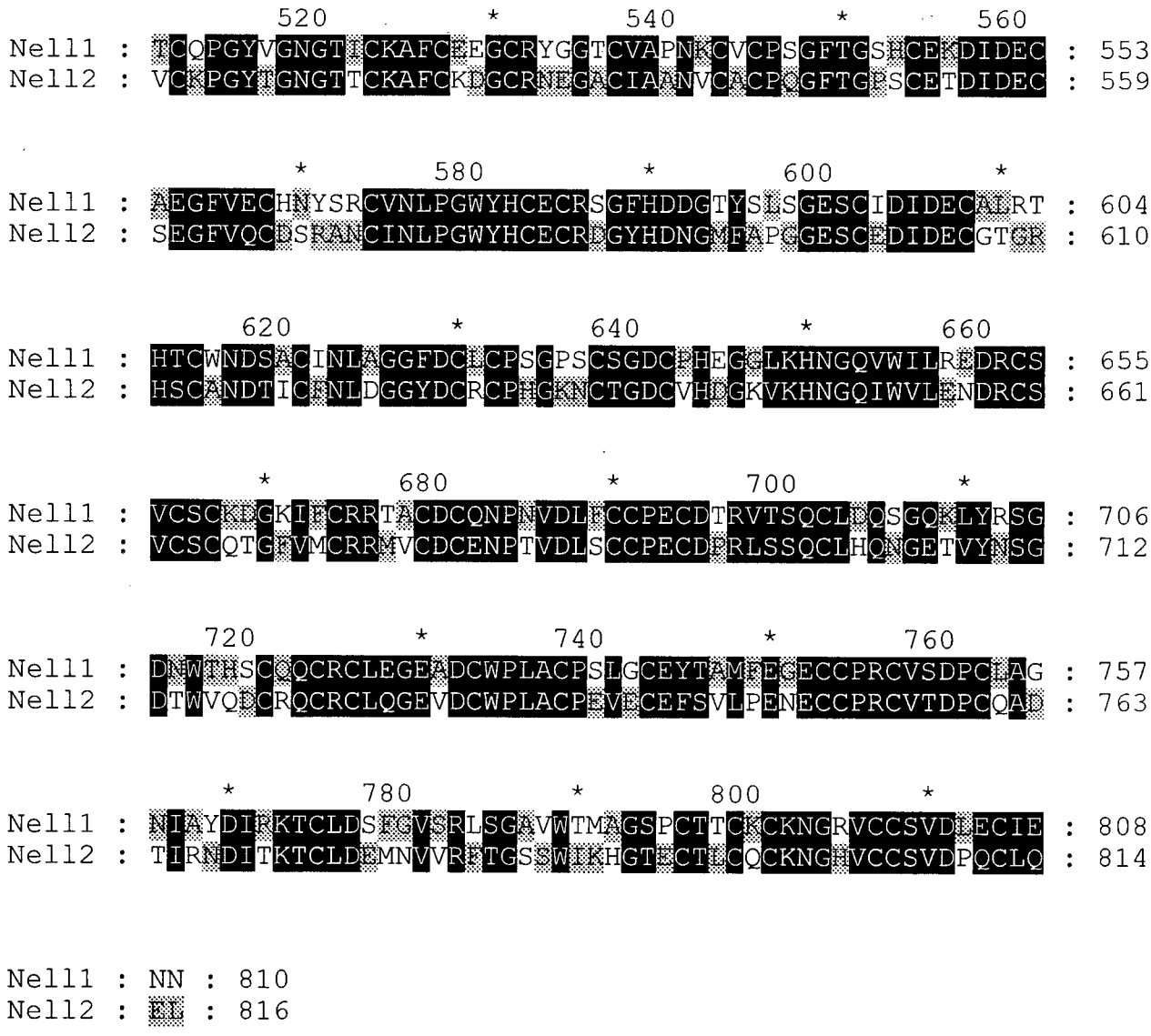
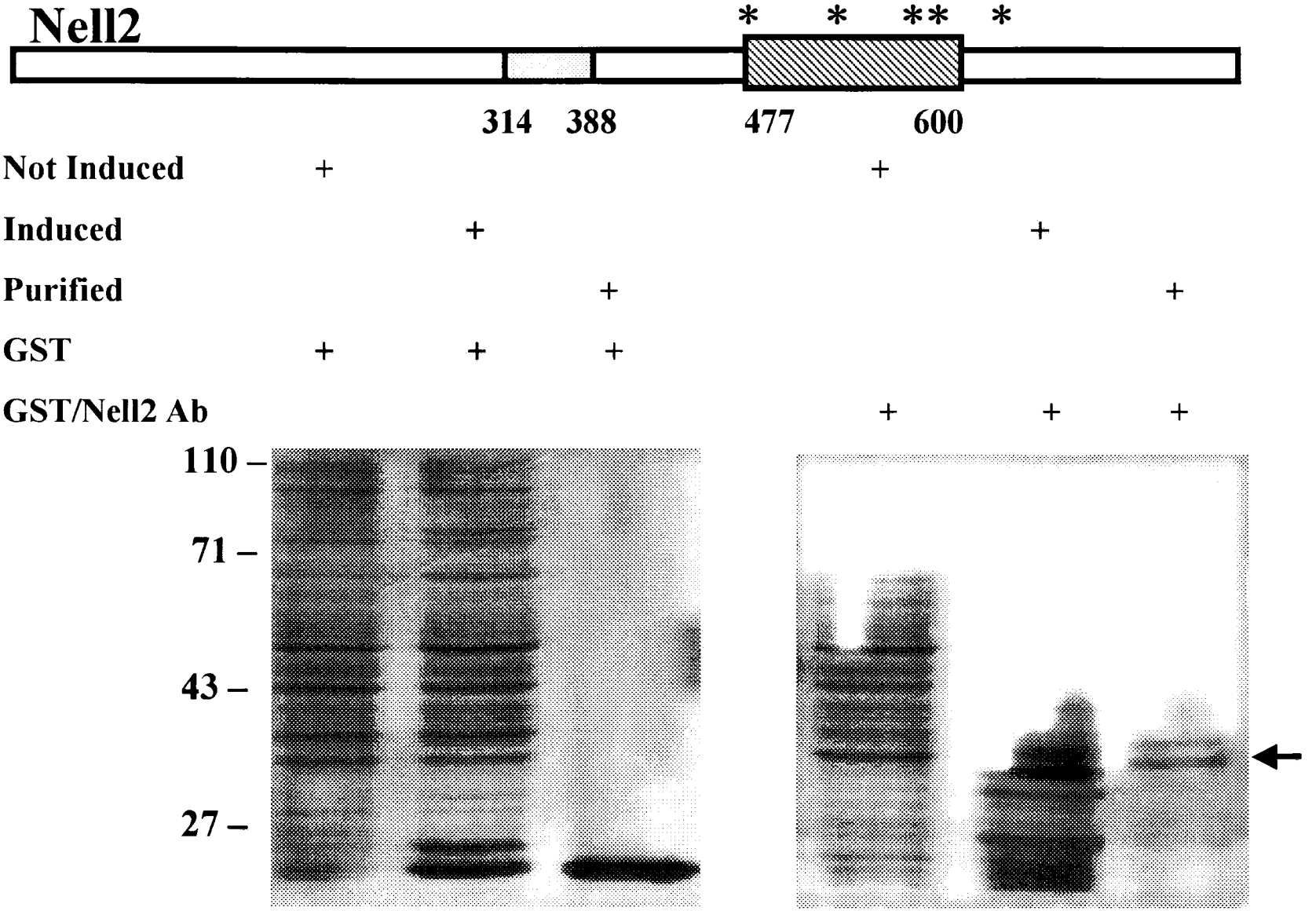

520 * 540 * 560
Nell1 : TCQPGYVGNGTICKAFCEEGCRYGGTCVAPNKCVCPSGFTGSHCEKDIDEC : 553
Nell2 : VCKPGYTGNGTTCKAFCKDGCRNEGACIAANVCACPCGFTGPSCETDIDEC : 559
* 580 * 600 *
Nell1 : AEGFVECHNYSRCVNLPGWYHCECRSGFHDDGTYSLSGESCIDIDECALRT : 604
Nell2 : SEGFVQCDSRANCINLPGWYHCECRDGYHDNGMFAPCGESCEDIDECGTCR : 610
620 * 640 * 660
Nell1 : HTCWNDSACINLAGGFDCLCPSGPSCSGDCPHEGCLKHNGQVWILREDRCS : 655
Nell2 : HSCANDTICRNLDGGYDCRCPHGKNCTGDCVHDGKVKHNGQIWVLENDRCS : 661
* 680 * 700 *
Nell1 : VCSCKDGKIECRRTACDCQNPNVDLECCPECDTRVTSQCLQQSGQKLYRSG : 706
Nell2 : VCSCQTGFVMCRRMVCDCENPTVDLSCCPECDPRLSSQCLHQNGETVYNSG : 712
720 * 740 * 760
Nell1 : DNWTHSCQQCRCLEGEADCWPLACPSLGCEYTAMPEGECCPRCVSDPCLAG : 757
Nell2 : DTWVQDCRQCRCLQGEVDCWPLACPEVECEFSVLPENECCPRCVTDPCQAD : 763
* 780 * 800 *
Nell1 : NIAYDIRKTCLDSFGVSRLSGAVWTMAGSPCTTCKCKNGRVCCSVDLECIE : 808
Nell2 : TIRNDITKTCLDEMNVVRETGSSWIKHGTECTLCQCKNGHVCCSVDPQCLQ : 814
Nell1 : NN : 810
Nell2 : EL : 816

Figure 20. Nell2 GST Fusion Results.

Top, schematic showing the location of the epitope target to Nell2 (314 to 388 aa) in relationship to the EGF-like repeats (*) and the predicted α_{1B} binding site (477 to 600 aa).

Bottom, acrylamide gel stained with Commassie blue showing E. coli extracts of cells expressing control GST (26 kDa) with the GST/Nell2 antigen fusion (36 kDa). Comparing the non-induced (no IPTG) and induced culture samples, the appearance of bands in the induced lane indicate increased protein expression. Samples were isolated and purified as described in Materials and Methods. The arrow indicates the GST/Nell2 antigen purified from bacteria and used for injection into rabbits. The lower band most likely represents a degraded product of the upper band.


Nell2
* * ** *
314 388 477 600
Not Induced + +
Induced + +
Purified + +
GST + + +
GST/Nell2 Ab + + +
110 –
71 –
43 –
27 –

virus 40 (SV40) large tumor (T) antigen which assists in protein expression from plasmids containing CMV promoters (Chang et al., 1985, Lebkowski et al., 1985). The HEK 108 cells stably express cloned N-type calcium channels (α_{1B}, β_{1b} and $\alpha_2\delta$ subunits; Stea et al., 1995, Bourinet et al., 1996). Detection of Nell2 was carried out by fluorescence immunostaining using the anti-Nell2 antibody with secondary anti-rabbit conjugated to FITC (**Figure 22**). Both cell lines showed endogenous Nell2 protein expression, confirming the Southern blot results of RT-PCR (**Figure 18**). These results also suggest that Nell2 expression is not dependent upon expression of the N-type channel. The majority of Nell2 protein expression appears to be localized to the membrane and is not present in the cell nucleus.

To determine cellular localization of N-type channels and Nell2, N-terminally Flag-tagged Nell2 was expressed in HEK 108 cells expressing N-type channels. Immunostaining was carried out using a mouse monoclonal anti-Flag antibody and secondary anti-mouse conjugated to Texas Red to identify Nell2 protein expression (red). Staining for the N-type channel was performed with an affinity purified rabbit polyclonal anti-α_{1B} antibody and secondary anti-rabbit antibody conjugated to FITC (green). Using confocal imaging both N-type channels and N-terminally Flag-tagged Nell2 expression was found to overlap (yellow) along the membrane of the cell body and extending processes (**Figure 23**).

To further analyze N-type calcium channel and Nell2 localization in HEK 293 cells, N-terminally Flag-tagged Nell2 and GFP tagged N-type calcium channel (α_{1B} GFP C-terminal fusion) were co-expressed in HEK 201 cells and immunostaining and confocal microscopy were performed. Nell2 protein expression was visualized with the mouse monoclonal anti-Flag antibody and secondary anti-mouse Texas Red (red) and N-type calcium channels were visualized using GFP (green). Again, results indicate both Nell2 and N-type channels co-

Figure 21. Antibody Staining of HEK Cell Expressed Nell2 .

HEK 201 cells were transiently transfected with Flag-tagged Nell 2 (Flag/Nell2) or Nell2 or pGemTE (Untransfected) and membrane and cytosolic fractions were separated by SDS electrophoresis. Western blots were carried out using polyclonal rabbit anti-Nell2 antibody purified against GST immunoreactivity (A) or affinity purified against the GST/Nell2 antigen (B). Secondary anti-rabbit and ECL staining were carried out as described in Materials and Methods. Positive control was the GST fusion antigen shown with small arrows (molecular weight = 36 kDa). Large arrows indicate the full length Nell2 expression in HEK 201 cells from pellet extracts using GST-purified anti-Nell2 antibody. Nell2 has a predicted moleulcar weight of 92 kDa and was usually detected at 110 kDa on Western blots. Non-specific binding was present in a 71 kDa band in both blots. The affinity purification process against the GST/Nell2 antigen resulted in loss of antibody immunoreactivity, therefore crude sera was used in all other experiments.

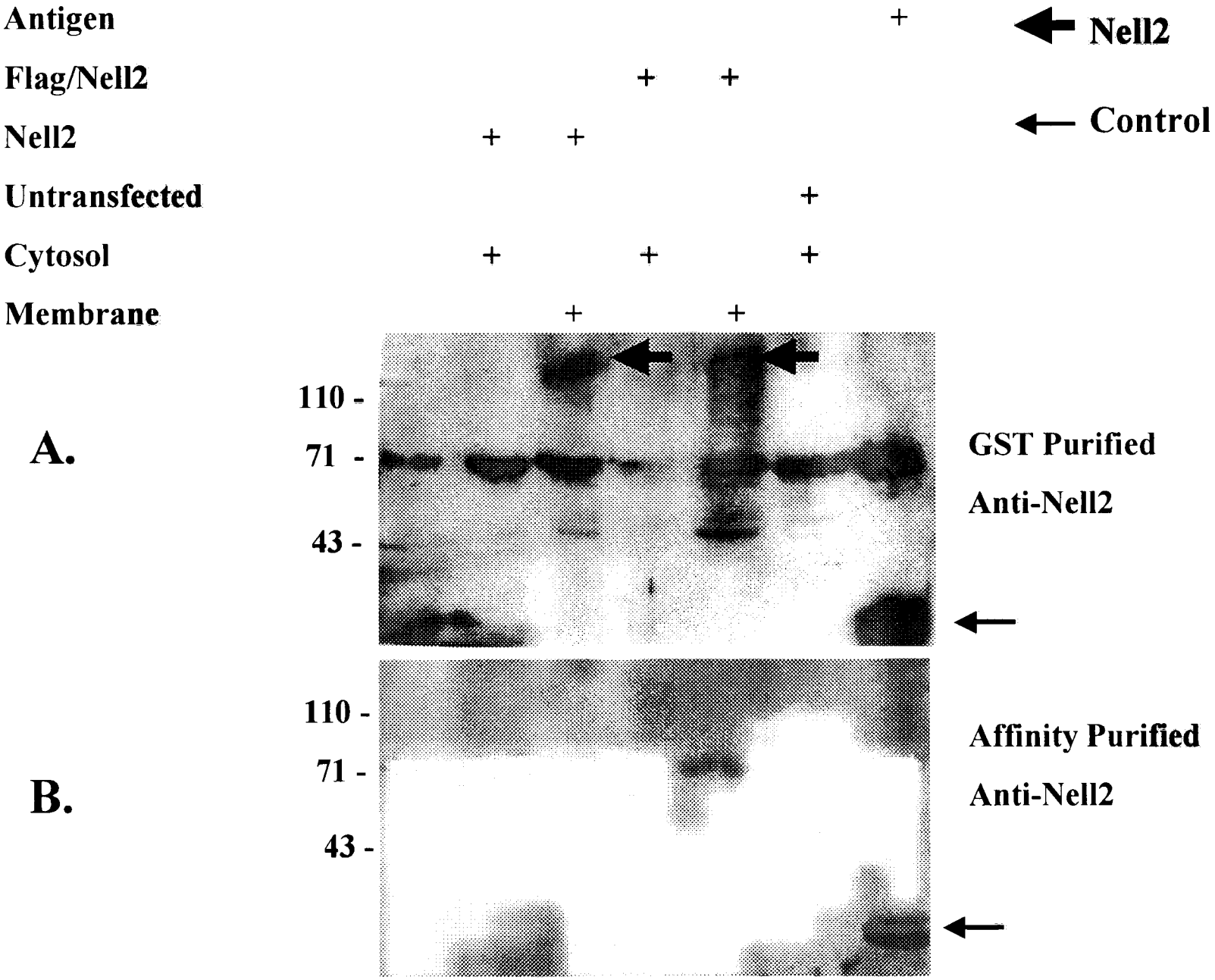
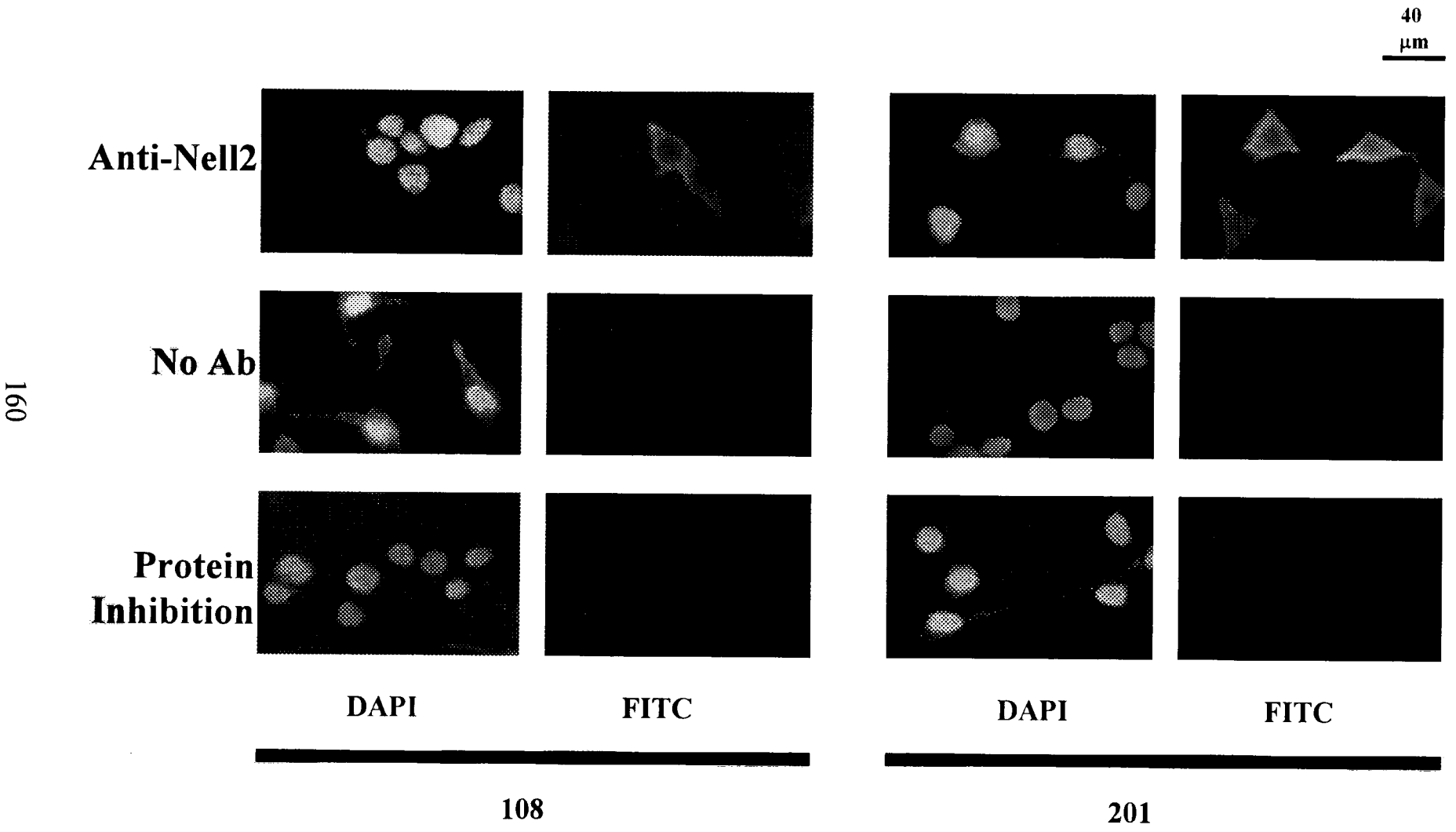

Antigen
+
Nell2
Flag/Nell2
+ +
Nell2
+ +
Control
Untransfected
+
Cytosol
+ + +
Membrane
+ +
A.
110 -
71 -
43 -
GST Purified
Anti-Nell2
B.
110 -
71 -
43 -
Affinity Purified
Anti-Nell2

Figure 22. Endogenous Nell2 Immunofluoresence in HEK Cells.

HEK cells (108 and 201) were stained for endogenous Nell2 with polyclonal rabbit anti-Nell2 antibody and visualized with a fluorescence microscope. Staining was not observed when no antibody or when fusion protein antigen was present in the primary buffer incubation. DAPI stains the nucleus of cells. Scale bar represents approximately 40 μm.


40
μm
Anti-Nell2
No Ab
Protein
Inhibition
DAPI
FITC
DAPI
FITC
108
201
160

localized to the plasma membrane in HEK 201 cells (**Figure 24**). Overlap in expression was observed along the entire cell including the processes. No nuclear staining of either protein was detected. Nell2 protein expression in HEK cells was examined further by determining localization within membrane and cytosolic fractions. Both HEK 201 and HEK 108 cell lines were transiently transfected with Nell2 or Flag-tagged Nell2 and fractions were separated by SDS gel electrophoresis. Western blot indicates that both proteins are predominantly expressed in the membrane fraction (**Figures 25 and 26**). These results confirm the cell staining patterns observed whereby Nell2 expression was cell membrane associated. Experiments examining HEK 201 cell extracellular solution did not indicate that Nell2 protein was being secreted (data not shown), in contrast to a previous report in transfected COS-7 cells (Kuroda et al., 1999).

A previous report suggested that the addition of a Flag epitope at the N-terminus disrupts expression of Nell2 in COS-7 cells (Kuroda et al., 1999). **Figure 25** shows Flag-tagged Nell2 was expressed in the membrane fraction of HEK cells and was approximately 110 kDa, similar to the *in vitro* translated Flag-tagged Nell2 using the Flag antibody. Thus in contrast to that for COS-7 cells, the N-terminal Flag epitope did not impede protein expression in HEK cells.

It should be noted that **Figure 26** shows a band in all lanes at approximately 110 kDa, which could represent endogenous Nell2 or non-specific background binding. The molecular weight of transfected Nell2 was always slightly larger (≈120 kDa) in HEK cells and also upon *in vitro* translation than the predicted molecular weight (92 kDa). In COS-7 cells, Nell2 was reported to be expressed as a glycosylated protein of 140 kDa, and a non-glycosylated protein of 90 kDa (Kuroda et al., 1999).

In neurons, N-type channels are localized predominantly to presynaptic terminals as well as

Figure 23. Confocal Double Label of Nell2/α_{1B} in HEK Cells.

HEK 201 cells were transiently transfected with α_{1B}, β_{1b} and $\alpha_2\delta$ subunits and N-terminal Flag-tagged Nell2 and stained for Nell2 and α_{1B} proteins by immunocytochemistry. Mouse anti-Flag M5 antibody was used with anti-mouse conjugated to Texas Red secondary (Texas Red staining identifies Nell2 expression). Anti-α_{1B} antibody was used with anti-rabbit conjugated to FITC secondary (FITC staining identifies N-type α_{1B} subunit expression). Overlap of Texas Red and FITC indicates Nell2/α_{1B} subunit expression appears most concentrated at the cell membrane and along the extending process. Scale bar represents approximately 10μm.

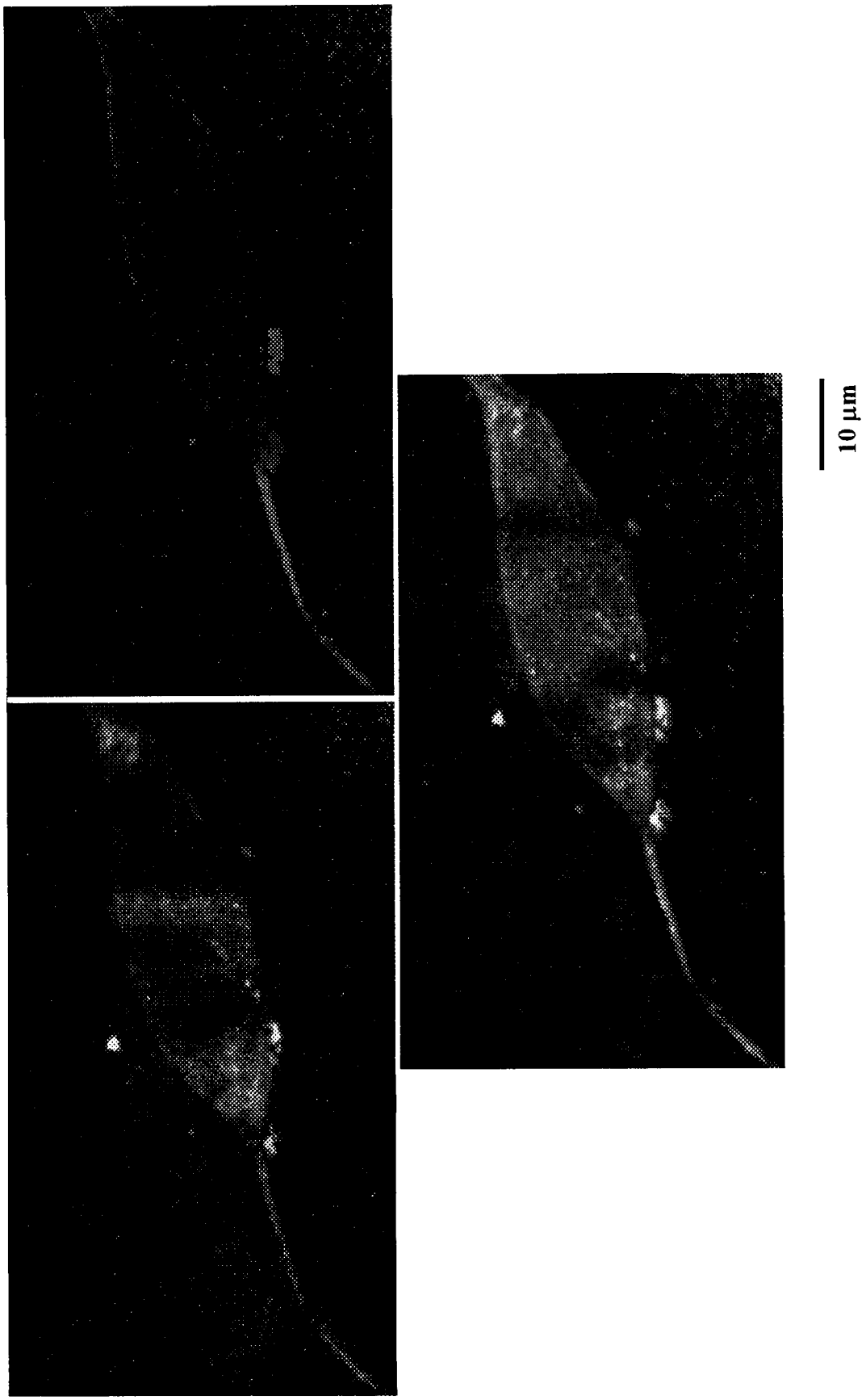

10 µm

Figure 24. Confocal Double Label of FlagNell2/GFP α_{1B} in HEK Cells.

HEK 201 cells were transiently transfected with C-terminal GFP-labeled α_{1B}, β_{1b} and $\alpha_2\delta$ subunits and N-terminal Flag-tagged Nell2. Cells were stained with mouse anti-Flag monoclonal antibody and anti-mouse conjugated to Texas Red secondary to show Nell2 protein expression. The overlap of GFP and Texas Red indicates localization of both the α_{1B} subunit and Nell2. Overlap of expression appears throughout the cell with the exclusion of the nucleus. Scale bar represents approximately 20 μm.


20 µm

Figure 25. Western blot of Flag/Nell2 Expressed in HEK Cells.

HEK 201 cells were transiently transfected with Flag-tagged Nell2. Membrane and cytosol fractions were separated and Western blots performed using monoclonal mouse M5 (anti-Flag) antibody followed by anti-mouse secondary and ECL as described in Materials and Methods. *In vitro* translated Flag-tagged Nell2 protein was used as a positive control. Flag-tagged Nell2 was predominantly expressed in the membrane fraction of HEK 201 cells, with a slightly larger molecular weight than predicted (110 kDa; arrow). Commassie stained gel demonstrates equal amount of protein was loaded for membrane and cytosol fractions. A modified Lowry assay confirmed the protein concentrations.

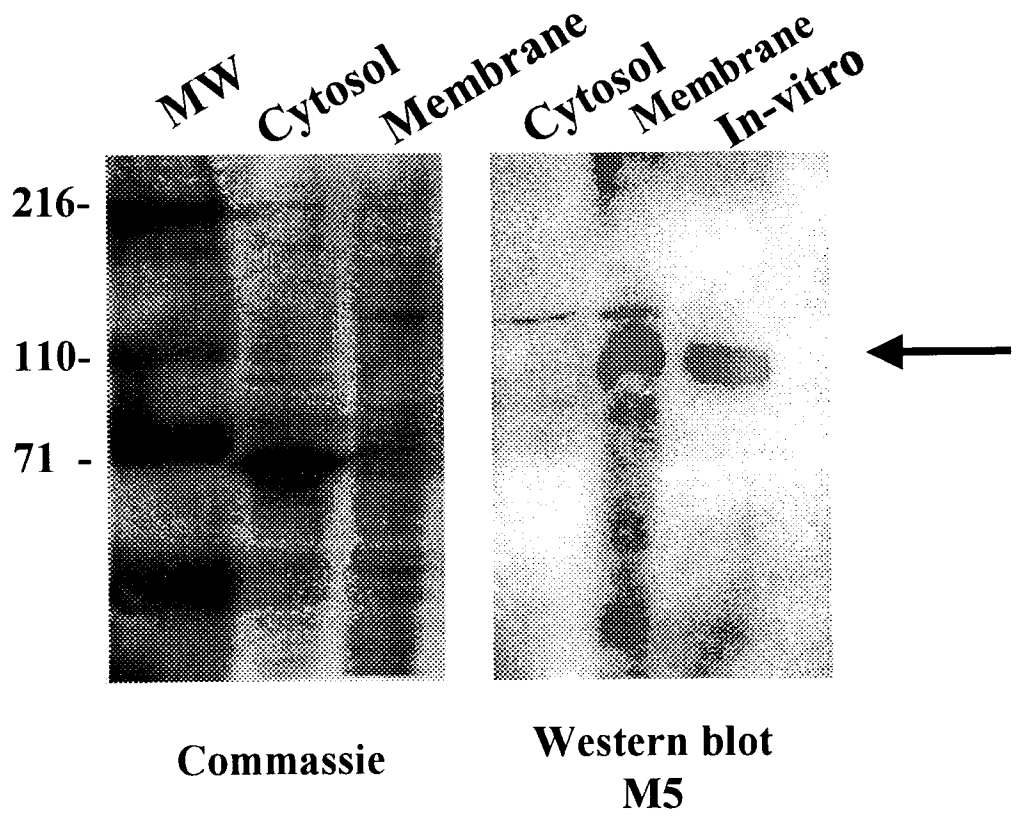
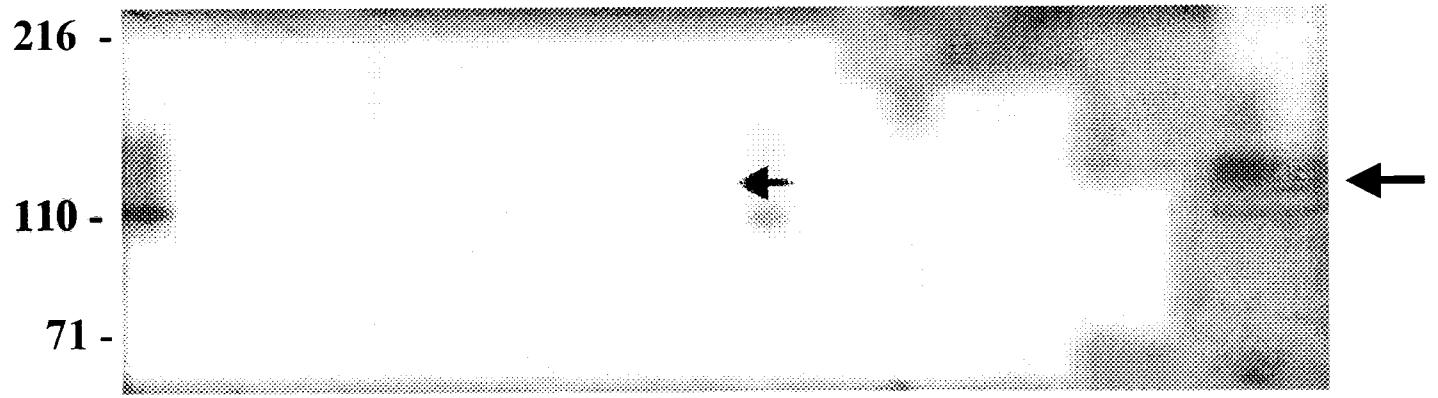

MW
Cytosol
Membrane
Cytosol
Membrane
In-vitro
216-
110-
71 -
Commassie
Western blot
M5

Figure 26. Western blot of Nell2 Expressed in HEK Cells.

HEK 201 and 108 cell lines were transiently transfected with Nell2 (Transfected) or carrier DNA (pGemTE, Non-transfected). The membrane and cytosol fractions were separated and Western blot performed using polyclonal rabbit anti-Nell2 antibody followed by anti-rabbit secondary incubation and ECL. Nell2 expression in HEK 201 and 108 cells was found predominantly in the membrane fraction (arrows).

108	+	+	+	+				
201					+	+	+	+
Cytosol	+	+			+	+		
Membrane			+	+			+	+
Non-transfected	+		+		+		+	
Transfecected		+		+		+		+



Anti-Nell2

postsynaptic dendrites (Westenbroek et al., 1992). Experiments were carried out on cultured adult rat primary cortical neurons to determine whether Nell2 protein colocalized to the same regions as N-type calcium channels in native cells. Endogenous staining was performed with polyclonal rabbit anti-Nell2 antibody using secondary anti-rabbit antibody conjugated to either rhodamine (red), or FITC (green). Nell2 appeared ubiquitously expressed in the cell body, axons and dendrites, although membrane staining of the cell body was observed by fluorescence microscopy (**Figure 27**). This result suggests that Nell2 could localize to the same subcellular regions as N-type channels in transfected HEK cells and primary cortical neurons.

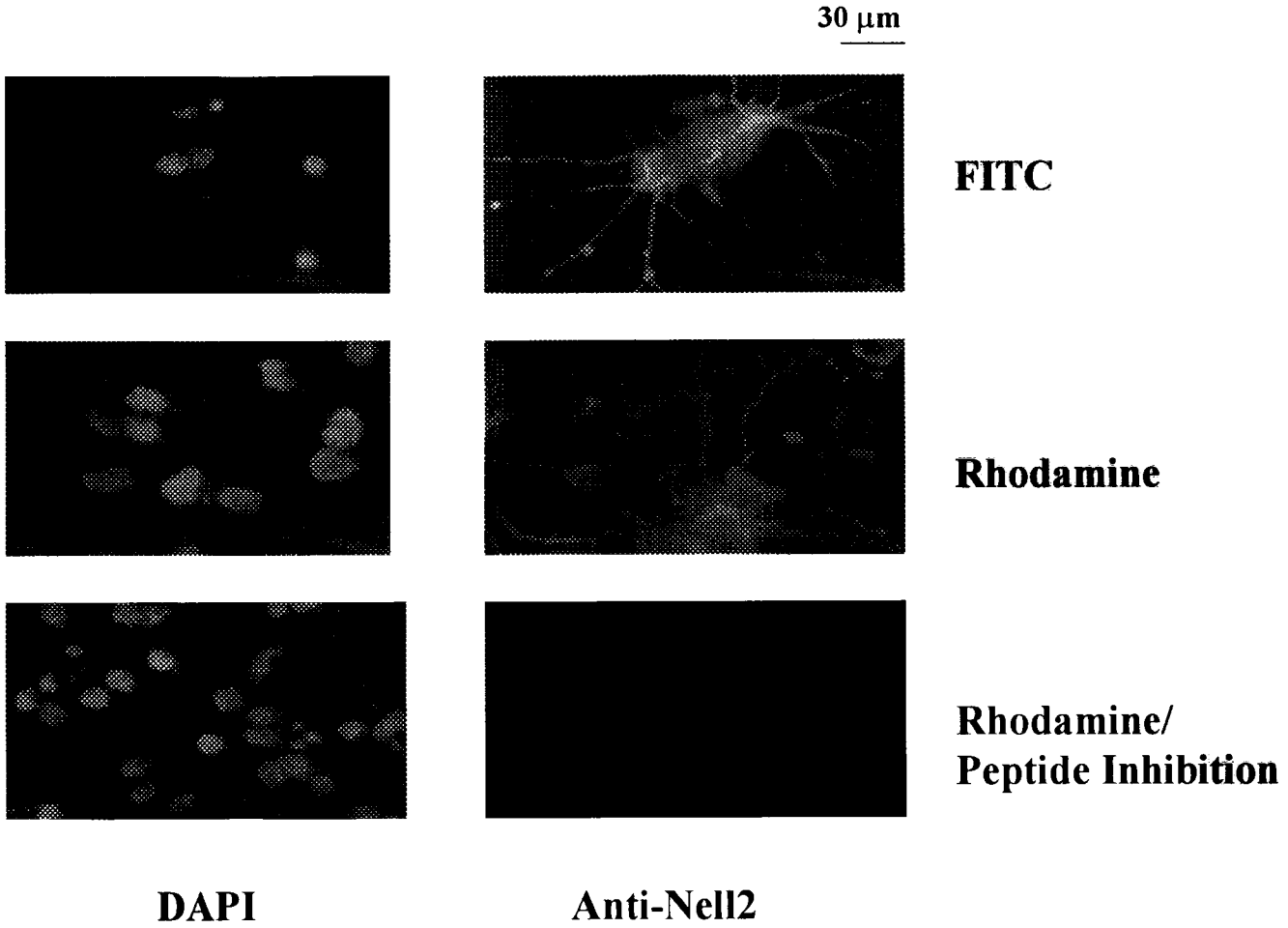
Nell2/N-type Channel Interaction

In order to demonstrate an *in vitro* protein-protein interaction between Nell2 and the N-type α_{1B} subunit, several attempts using a variety of binding protocols were performed. First, GST fusion proteins were generated with two of the yeast two-hybrid clones (partial Nell2 sequences), expressed in E. coli and whole cell lysate was incubated with *in vitro* translated ^{35}S-methionine labeled Bait 4 (C-terminus of the α_{1B} subunit). Proteins were isolated on GST resin and SDS electrophoresis was carried out. Proteins were then blotted to Whatman paper and exposed to autoradiographic film. GST fusion proteins isolated from E. coli were also immobilized on nitrocellulose by slot blot apparatus and probed with *in vitro* translated ^{35}S-methionine labeled Bait 4. The variables examined with E. coli expressed protein included different protein concentrations, buffers, blocking agents and calcium and potassium gradients (**Figures 28 to 30**).

In other experiments, whole cell lysates of HEK cells expressing full-length and partial fragments of Nell2 were incubated with *in vitro* translated ^{35}S-methionine-labeled Bait 4 and gel shift assays were performed using native polyacrylamide gel electrophoresis. Co-immunoprecipitations were also carried out using a variety of Nell2 constructs and epitope tags

Figure 27. Staining for Nell2 in Primary Cortical Neurons.

Rat cultured primary cortical neurons were stained for endogenous Nell2 with polyclonal rabbit anti-Nell2 antibody and visualized with a fluorescence microscope. Secondary staining included rhodamine or FITC. Incubation of fusion protein antigen in the primary incubation inhibited staining. Nell2 staining appears dispersed throughout the neuron, including the cell body, axons and dendrites.


30 μm
FITC
Rhodamine
Rhodamine/
Peptide Inhibition
DAPI
Anti-Nell2

in HEK cells and with *in vitro* translated proteins. Yeast two-hybrid clones and Bait 4 were myc- and His-tagged and Nell2 and Bait 4 were N-terminally Flag-tagged. Both Protein A and Protein G resins were used in combination with mouse and rabbit antibodies. Some variables for co-immunoprecipitation experiments included tissue culture cell transfection protocols, protein expression and binding incubation times, lysis and binding buffers, detergents, protease inhibitors, salt concentration, calcium concentration, protein concentration, primary and secondary antibodies used and antibody concentrations.

Although many different experiments were carried out, only the nickel resin co-immunoprecipitation results will be shown here. Nell2 or N-terminally Flag-tagged Nell2 proteins were expressed by *in vitro* translation (**Figure 28**) or in HEK cells and Bait 4 was C-terminally histidine tagged and *in vitro* translated using ^{35}S-methionine. The optimal conditions for elution of Bait 4 off nickel resin were determined and included 60 mM imidazole (**Table 8, Figures 29 and 30**).

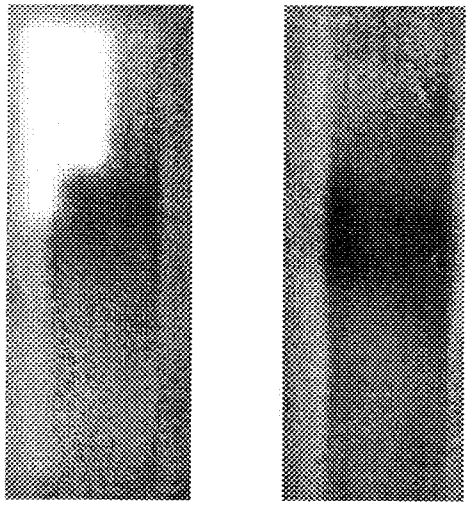
Under the conditions used in this experiment and all of the others described above, Bait 4 and Nell2 were not found to interact *in vitro* (**Figure 31**). The yeast two-hybrid system has been shown to detect weak and transient interactions that other techniques may not be sufficiently sensitive to detect and therefore the typical protocols for demonstrating a protein-protein interaction may not be adequate for the Nell2/α_{1B} interaction. The experimental limitations with *in vitro* binding methods may suggest some yeast two-hybrid findings are physiologically irrelevant. One must consider the nature of transient interactions which show fast association/dissassociation kinetics and may therefore be more adaptable to rapid changes in the environment, particularly in the presynaptic terminal and events leading to neurotransmission.

Figure 28. *In vitro* Translation of Nell2 and Flag-tagged Nell2.

A. Autoradiogram of cloned rat Nell2 and Flag-tagged Nell2 *in vitro* translated with ^{35}S-methionine.

B. Western blot of cloned rat Nell2 and Flag-tagged Nell2 *in vitro* translated without label. Primary monoclonal mouse M5 (anti-Flag) antibody was used on the Flag/Nell2 blot and rabbit polyclonal anti-Nell2 antibody was used on the Nell2 blot. Secondary antibody incubation and ECL were carried out as described in Materials and Methods. The predicted molecular weight of Nell2 was 92 kDa although Nell2 always expressed at a slightly larger molecular weight (ranging from 110 to 120 kDa).

A. Autoradiogram



B. Western Blot

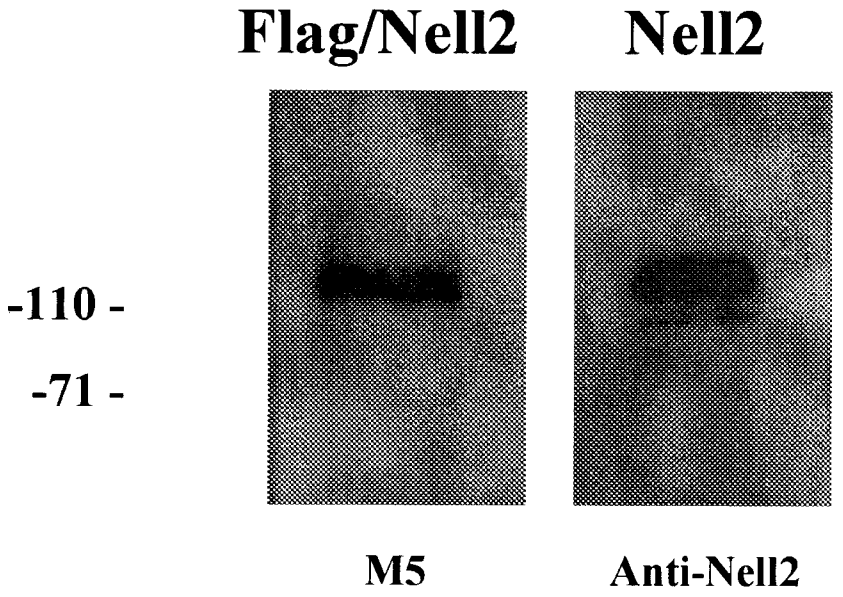


Figure 29. Imidazole Elution of Bait 4 Fusion Protein.

^{35}S-methionine labeled *in vitro* expressed His-tagged Bait 4 was eluted off Ni^{2+}-agarose beads at 60 to 100 mM imidazole. The optimal concentration of imidazole for elution was determined to be 80 mM. Nell2 did not bind or elute from Ni^{2+}-agarose beads (negative control).

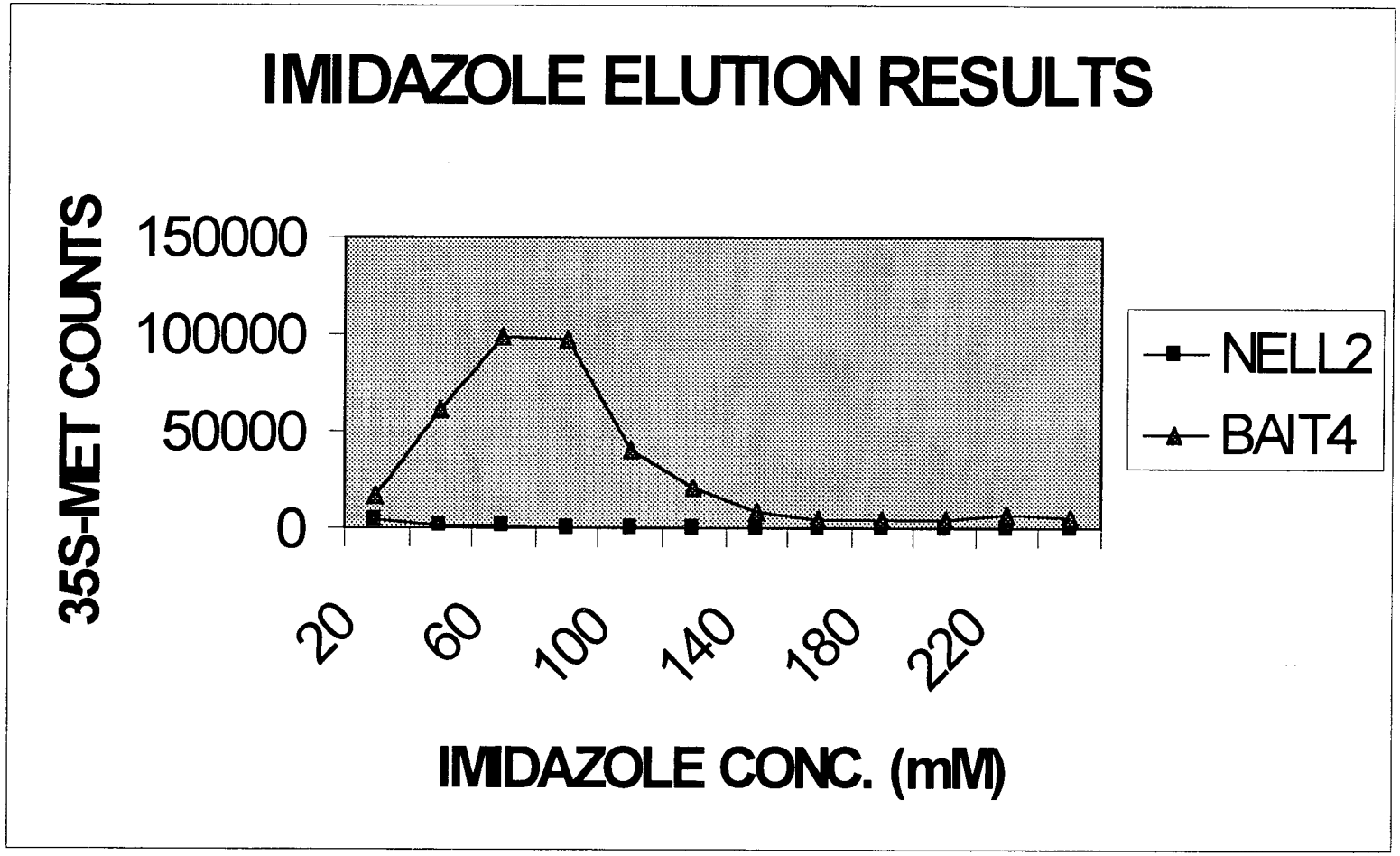

IMIDAZOLE ELUTION RESULTS
35S-MET COUNTS
150000
100000
50000
0
20
60
100
140
180
220
IMIDAZOLE CONC. (mM)
NELL2
BAIT4

Figure 30. pH Elution of Bait 4 Fusion Protein.

^{35}S-methionine labeled *in vitro* expressed His-tagged Bait 4 was eluted off Ni^{2+}-agarose beads initially with a pH of 7.8 to 7.4 as well as pH lower than 5.5. Nell2 did not bind or elute from Ni^{2+}-agarose beads (negative control).

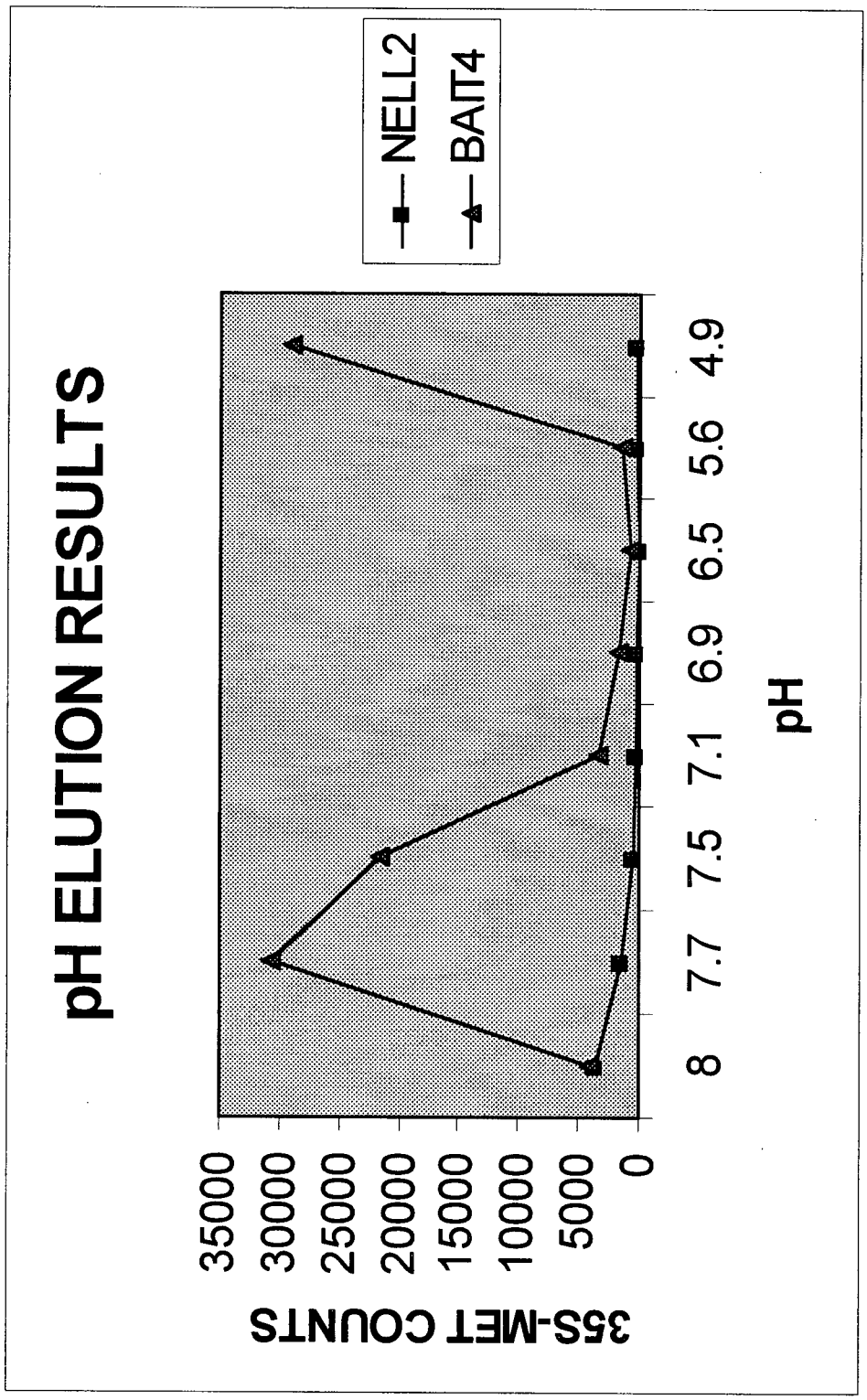

pH ELUTION RESULTS
NELL2
BAIT4
35000
30000
25000
20000
15000
10000
5000
0
35S-MET COUNTS
8
7.7
7.5
7.1
6.9
6.5
5.6
4.9
pH

Figure 31. Nell2/Bait 4 Binding Studies with Nickel.

^{35}S- methionine labeled *in vitro* translated proteins were bound to pre-washed Ni^{2+}-agarose resin and eluted with 80 mM imidazole and visualized by autoradiography. Bait 4 was C-terminally tagged with His and demonstrated to bind the resin efficiently, eluting at 80 mM imidazole. Nell2 did not to bind to the resin. Bait 4 and Nell2 probes were controls showing expression of proteins in the *in vitro* reactions used in the pull down experiment. Eluted Bait 4, Nell2 and Bait 4 + Nell2 were samples collected after addition of 80 mM imidazole. The conditions of this experiment were not optimal for Nell2/Bait 4 protein interaction.

MW (kDa)
BAIT 4 PROBE
NELL2 PROBE
ELUTED BAIT 4
ELUTED NELL2
ELUTED BAIT 4
+ NELL2
110 -
71 -
43 -

Table 10. Summary of Nickel Binding Controls.

Gradients were established in determining the optimal concentration of imidazole or optimal pH for elution of ^{35}S-methionine labeled *in vitro* expressed His-tagged Bait 4 off of Ni^{2+}-agarose beads. Imidazole was tested at a range of 20 to 240 mM (A) and pH ranged from 8.0 to 5.6 (B). Elution values were determined by scintillation counting. Nell2 was used as a control.

A. Determination of Optimal Imidazole Concentration for Elution.

Imidazole Concentration (mM)	^{35}S-Met Counts for Nell2	^{35}S-Met Counts for Bait 4
20	4770	16570
40	1802	61068
60	1111	98658
80	525	97216
100	374	39941
120	368	20862
140	220	8009
160	170	3847
180	121	4094
200	113	4279
220	118	6270
240	79	5503

B. Determination of Optimal pH for Elution.

pH	^{35}S-Met Counts for Nell2	^{35}S-Met Counts for Bait 4
8.0	3656	4032
7.7	1409	30743
7.5	468	21630
7.1	247	3391
4.9	136	1675
6.9	79	751
6.5	120	1368
5.6	308	28901

Chapter 5. Functional Interaction Between Nell2 and the N-type Calcium Channel

Background

Protein phosphorylation is a key regulator of cellular activity and plays a significant role in calcium channel physiology (Armstrong and Eckert, 1987). Rat Nell2 was first isolated by the yeast two-hybrid system by screening a rat expression library with a PKC βI regulatory region (Kuroda et al., 1999). Nell2 was later shown to bind other PKC isoforms, including a strong interaction with the ε and ζ isoforms and a weak interaction with γ and δ (Kuroda and Tanizawa, 1999). With this in mind, it is possible that Nell2 may help localize PKC to N-type channels to facilitate rapid modulation, similar to the role that AKAP79 plays in modulation of L-type channels (Johnson et al., 1994, Gao et al., 1997).

To date, eleven PKC isoforms have been described and are grouped according to their N-terminal structures. The typical class includes the α, β (I and II) and γ isoforms. The novel class includes δ, ε, η, θ and μ and the atypical class includes the ζ and ι/λ isoforms. The typical PKC isoforms are regulated by calcium, phorbol esters or diacylglycerol and phorbol esters bind to the C1 region of both typical and novel PKC isoforms. The novel PKC isoforms are calcium independent but can be stimulated by diacylglycerol still. The atypical isoforms do not bind phorbol esters (calcium and phosphodiesterase independent) (Huang and Huang, 1993).

The phorbol ester 12-myristate-13-acetate (PMA) is known to activate the PKC pathway in many

cell types (Mosior and Newton, 1995). Activation of the PKC pathway can cause upregulation of some calcium channels, including L-, R- and N-types (Yang and Tsien, 1993, Stea et al., 1995). Activation of PKC pathway has also been shown to inhibit some calcium channel currents (Rane and Dunlap, 1986, Hammond et al., 1987, Rane et al., 1989, , Redman et al., 1997, Hasson, 1998). Additionally, some phorbol esters have been shown to reduce calcium currents independent of PKC activation (Hockberger et al., 1989, Doerner et al., 1990).

In *Xenopus oocytes*, PMA induced the upregulation of both cloned α_{1B} and α_{1E} channels (30 to 40 % increase in peak currents), but not α_{1A} or α_{1C} channels when co-expressed with the β_{1b} subunit (Stea et al., 1995). The upregulation was dependent upon co-expression of the α_1 subunit with the β subunit (Stea et al., 1995). PMA also shifted the current voltage relations of α_{1E} and α_{1B} currents and both activation and inactivation rates for α_{1E} were slowed but were not affected in the α_{1B} channel.

Another intracellular modulator of calcium channel activity are the heterotrimeric G-proteins. The native R-, P/Q- and N-type calcium channels are modulated by G-proteins, resulting in pronounced voltage-dependent inhibition (Dunlap and Fischbach, 1981, Swartz, 1993, Swartz et al., 1993, Hille, 1994, Stea et al., 1995, Bourinet et al., 1996). Studies with the cloned channels show activation of the G-protein pathway leads to a reduced peak current and slowing of the inactivation and activation kinetics, causing a positive shift in voltage dependence of channel activation and a shift in current-voltage (I-V) relationship (Bean, 1989, Zamponi and Snutch, 1998b). This G-protein inhibition is defined by a strong voltage-dependence characterized by a prepulse-dependent facilitation (Marchetti et al., 1986; Tsunoo et al., 1986, Elmslie et al., 1990; Ikeda, 1991).

The $G_{\beta/\gamma}$ subunits appear to be largely responsible for channel inhibition and the target site of the interaction is thought to be mainly the I-II linker region of the α_{1B} subunit, although the N-terminus including domain I and the C-terminus have also been implicated (Zhang et al., 1996, De Waard et al., 1997, Herlitze et al., 1997, Qin et al., 1997, **Table 11**). Direct binding of $G_{\beta/\gamma}$ has been demonstrated to the α_{1A}, α_{1B} and α_{1E} subunits, which all contain a $G_{\beta/\gamma}$ consensus binding motif (QXXER, De Waard et al., 1997). Native L-type currents from cardiac cells, smooth muscle, endocrine cells and neurosecretory cells also exhibit G-protein-dependent modulation (Reuter, 1983, Fischmeister and Hartzell, 1986, Dolphin, 1999, Viard et al., 1999). The mechanism of voltage-dependent inhibition appears to be mediated through second messengers like PKA or CaM, although $G_{\beta/\gamma}$ binding to α_{1C} has been demonstrated (Ivanina et al., 2000).

AKAPs anchor and localize kinases and phosphatases to sites of action and Nell2 is a potential AKAP-type protein since it has been shown to bind PKC and may also bind other signaling molecules (Kuroda and Tanizawa, 1999). The Nell2 protein sequence was searched for known G-protein binding consensus sequences but no significant homology was found, other than a domain common to G-protein coupled receptors (**Figure 13**).

Modulation of calcium channels via the G-protein pathway can be mimicked by co-expression of cloned calcium channels with a cloned metabotropic glutamate receptor (mGluR1α), which activates phospholipase C via a G-protein dependent mechanism (Masu et al., 1991, Nakamura et al., 1994). Activation of endogenous somatostatin receptors in HEK cells also initiates the G-protein pathway (Zamponi et al., 1997). Activated $G_{\beta/\gamma}$ results in a decrease in the first latency in response to depolarization (Patil et al., 1996, Zamponi and Snutch, 1998a). Dissociation of $G_{\beta/\gamma}$

Table 11. Identified G-protein Binding Sites on Cloned Calcium Channels.

$G_{\beta\gamma}$ and $G_{o\alpha}$ binding sites have been identified on various calcium channel α_1 subunits. 1. Zamponi et al., 1997, 2. Qin et al., 1996, 3. Dolphin et al., 1999, 4. Page et al., 1998, 5. Furukawa et al., 1998.

Interaction	Binding Site
Rat $\alpha_{1A}/G_{\beta\gamma}$	α_{1A}(416-434)[1]
Rat $\alpha_{1B}/G_{\beta\gamma}$	α_{1B} (410-428)[1]
Rat $\alpha_{1E}/G_{\beta\gamma}$	α_{1E} (356-451 and 2036-2074)[2]
Rat $\alpha_{1B}/G_{\beta\gamma}$	α_{1B} (1-55)[3]
Rat $\alpha_{1E}/G_{\beta\gamma}$	α_{1E} (1-50)[4]
Rabbit $\alpha_{1B}/G_o\alpha$	α_{1B} (1931-1949)[5]
Rabbit $\alpha_{1A}/G_o\alpha$	α_{1A}(2028-2046)[5]

is thought to occur with a strong depolarizing prepulse and results in an apparent facilitation of whole cell currents (Bean, 1989, Kasai, 1991, Elmslie et al., 1992, Patil et al., 1996).

The calcium channel β subunit antagonizes the inhibitory effects of $G_{\beta/\gamma}$ by crosstalk within the I-II linker region of the α_1 subunit (Zamponi and Snutch, 1998a, Zamponi and Snutch, 1998b). The $G_{\beta/\gamma}$ interaction also appears to undergo crosstalk with the PKC pathway in the same region of the N-type α_{1B} subunit (Zamponi et al., 1997, Hamid et al., 1999). For example, in contrast to the G-protein inhibition, PKC phosphorylation leads to upregulation of N-type channel activity. Two PKC phosphorylation sites within the I-II linker have been identified, one of which (Thr^{422}) mediates the antagonistic effect of PKC upregulation on G-protein inhibition of N-type calcium channels (Hamid et al., 1999).

Co-expression of N-type channels with syntaxin-1A has several functional effects which include a negative shift in inactivation, a slowing of inactivation kinetics and a large tonic G-protein inhibition mediated by $G_{\beta/\gamma}$ (Bezprozvanny et al., 1995; Bezprozvanny et al., 2000; Degtiar et al., 2000; Jarvis et al., 2000). Syntaxin-1A directly binds the α_{1B} Synprint site (Sheng et al., 1994; Yokoyama et al., 1997) and independently binds $G_{\beta/\gamma}$ (Jarvis et al., 2000). Syntaxin-1A binding to the Synprint site can be disrupted by PKC phosphorylation of residues within the Synprint site (Yokoyama et al., 1997). Furthermore, binding to the Synprint site or to the $G_{\beta/\gamma}$ subunit can be attenuated by interaction with the SNAP-25/nSec-1 complex (Hata et al., 1993; Pevsner et al., 1994, Jarvis and Zamponi, 2001). When syntaxin-1A is bound to the α_{1B} subunit, it is thought to localize and enhance tonic inhibition by $G_{\beta/\gamma}$ (Jarvis and Zamponi, 2001).

Cysteine string proteins bind the α_{1B} subunit, syntaxin-1A and the $G_{\beta/\gamma}$ subunit and enhance N-

type channel tonic G-protein inhibition (Leveque et al., 1998; Nie et al., 1999; Wu et al., 1999, Magga et al., 2000). One of the putative $G_{\beta/\gamma}$ subunit binding sites on the α_{1B} subunit is present in Bait 4, the interaction region of Nell2 in the yeast two-hybrid system and it is possible that Nell2 plays a role in both PKC and G-protein modulation of N-type channels.

Results

Nell2 and PKC-dependent Modulation of N-type Calcium Channels

PKC modulation of the N-type channel was carried out in collaboration with Dr. Arik Hasson (postdoctoral fellow in Dr. Terry Snutch's laboratory). Previous studies in HEK cells showed that application of PMA (100 to 400 nM) by perfusion caused a 50 to 60 % upregulation of transiently expressed N-type calcium channels (Zamponi et al., 1997, Hamid et al., 1999). Here, the nystatin whole cell patch clamp technique was used on a stable HEK cell line expressing N-type calcium channels (α_{1B}, $\alpha_2\delta$ and β_{1b} subunits) or transiently transfected HEK 201 cells using Ba^{2+} as the charge carrier. Peak upregulation was induced at 25 nM PMA applied via direct application in bath (**Figure 32**). The data suggests PMA has a time dependent biphasic effect in that it enhances N-type current amplitudes (I_{Ba}) at PMA concentrations between 5 and 200 nM and inhibits I_{Ba} at higher concentrations. Above 75 nM, PMA mainly decreased I_{Ba} and above 200 nM the effect of PMA was a large stable inhibition (60 to 100 %) compared with that of control. These effects were not observed when using an inactive isoform of PMA (0.5 μM 4-α-PMA) and were completely blocked with 100 nM calphostin C, a PKC inhibitor (data not shown). The time course of applying 25 nM PMA by bath application to HEK cells expressing N-type channels first displayed a sharp decrease in I_{Ba} followed by a marked increase, hence a time dependent, biphasic effect (**Figure 33**).

Figure 32. Biphasic PMA Effect on N-type Channels.

N-type calcium channels were stably expressed in HEK cells (108) and whole cell currents measured by the perforated whole cell patch clamp technique (0.1 % nystatin) using Ba^{2+} as the charge carrier. Current traces were induced by stepping the cells from -100 to +20 mV. PKC activation was carried out by addition of phorbol ester, β-Phorbol 12 myristate-13-acetate (PMA) by bath application. The maximal up-regulation of wild type N-type channels (50 to 85 % increase) by PKC activation was reached by 25 to 100 nM PMA, while lower concentration of PMA (2.5 nM) had a very mild effect. The PMA effect on HEK expressed N-type channels was time dependent, biphasic and concentration dependent. Higher concentrations of PMA (200 nM) leads to a decrease in current amplitude. See **Figure 33** for description of time course of miximal inhibition and enhancement.

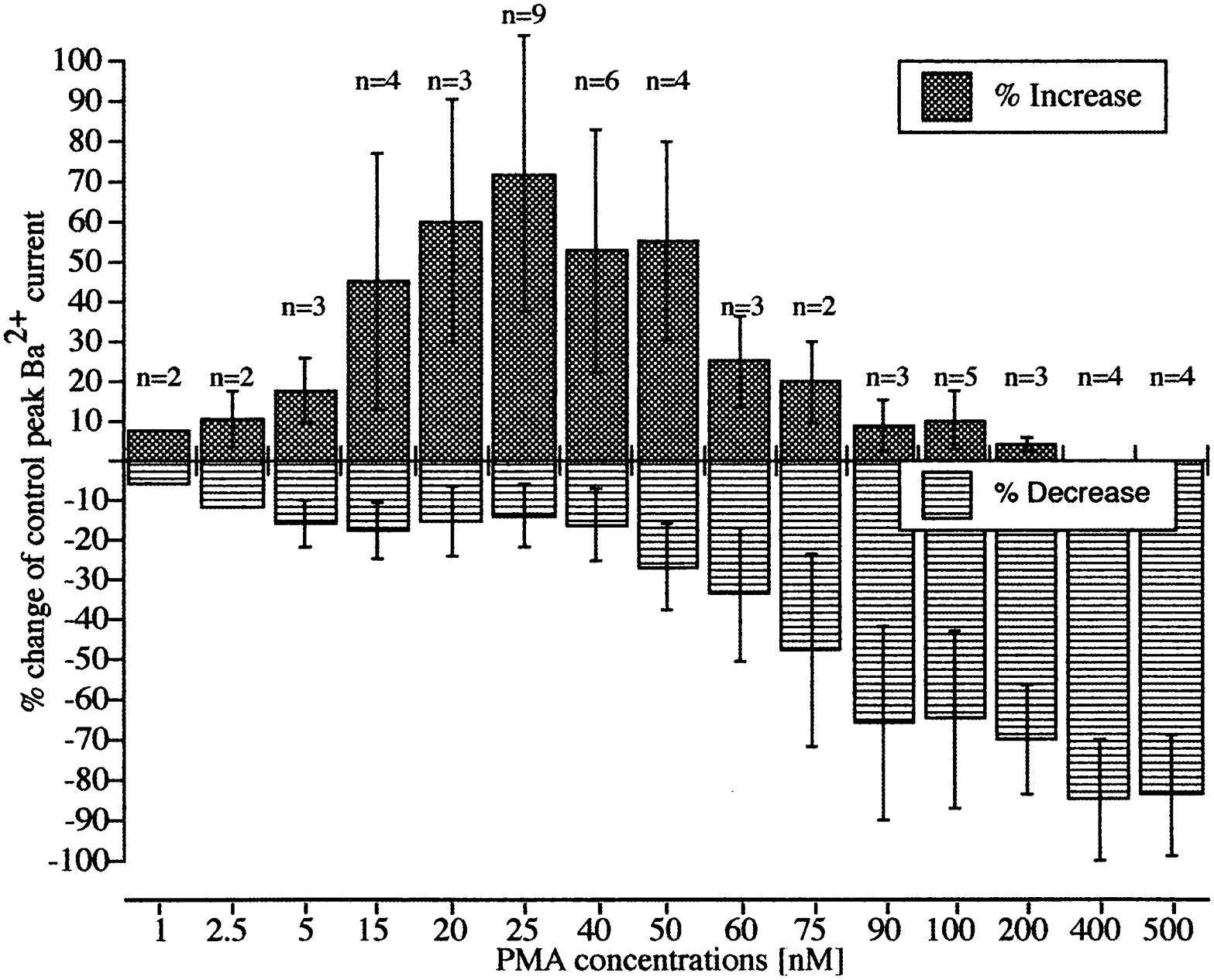
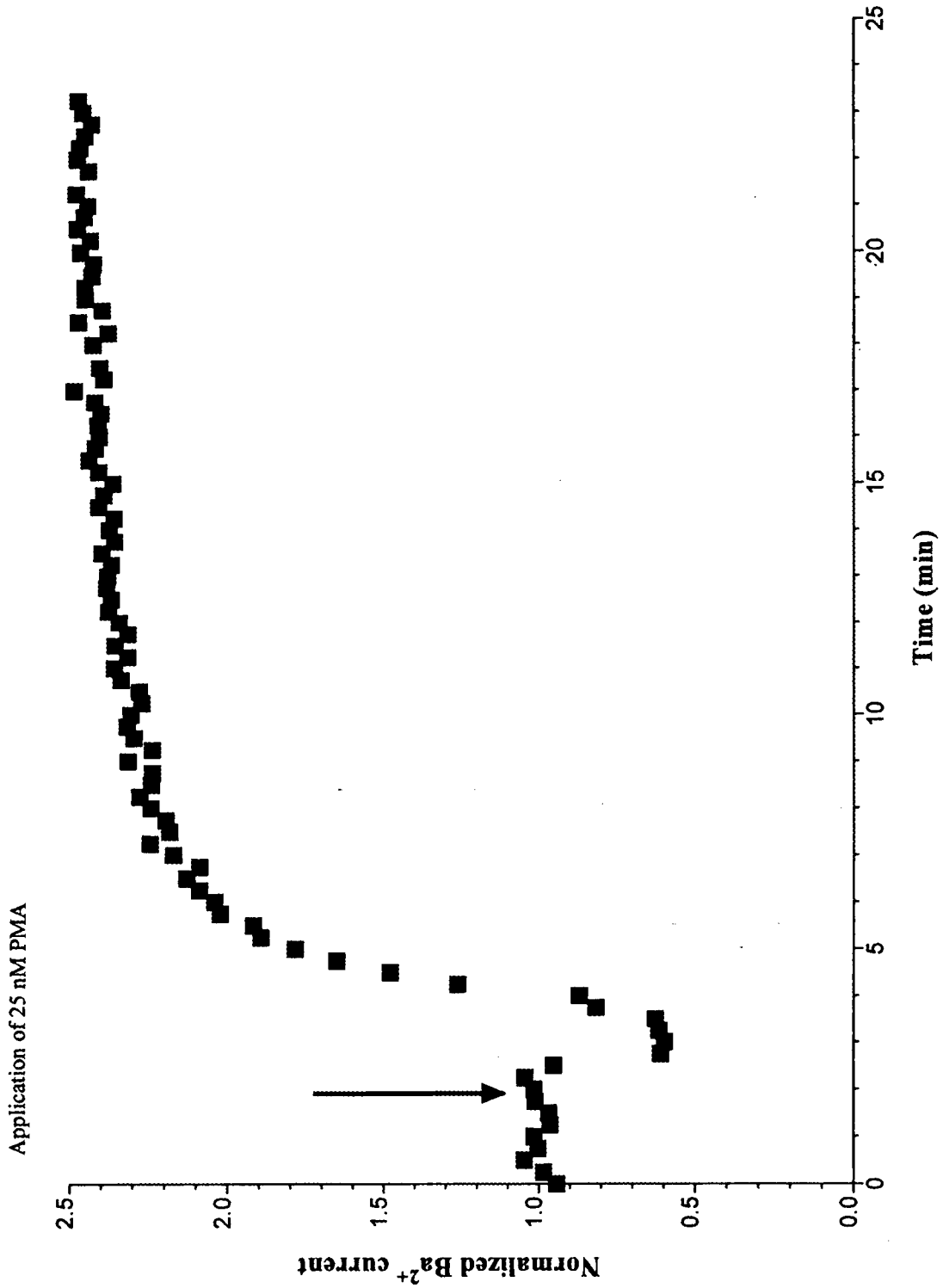

% Increase
% Decrease
% change of control peak Ba^{2+} current
100
90
80
70
60
50
40
30
20
10
-10
-20
-30
-40
-50
-60
-70
-80
-90
-100
n=2
n=2
n=3
n=4
n=3
n=9
n=6
n=4
n=3
n=2
n=3
n=5
n=3
n=4
n=4
1
2.5
5
15
20
25
40
50
60
75
90
100
200
400
500
PMA concentrations [nM]

Figure 33. Time Course of PMA Effect on N-type Channels.

N-type calcium channels were stably expressed in HEK cells (108) and currents were measured by the perforated whole cell patch clamp technique (0.1 % nystatin) using Ba^{2+} as the charge carrier. Current traces were induced by stepping the cells from -100 to +20 mV. The time course was normalized to the current peak amplitude before PMA application and at the indicated time 25 nM PMA was applied. Bath application of PMA causes an initial decrease in current amplitude that is followed by an increase in current amplitude.


Application of 25 nM PMA
Normalized Ba^{2+} current
Time (min)
0.0
0.5
1.0
1.5
2.0
2.5
0
5
10
15
20
25

Interestingly, when Nell2 was co-expressed with N-type channels in HEK cells, 2.5 nM PMA resulted in a similar increase in I_{Ba} to that observed with N-type channels alone with 25 nM PMA (**Figure 34**). It thus appears that N-type calcium channels are more sensitive to PMA when co-expressed with Nell2, shifting the dose response for PMA leftward (data not shown). Co-expression of antisense Nell2 in HEK cells expressing N-type channels abolished the observed 25 nM PMA induced increase in I_{Ba} (**Figure 35**). The loss of current increase ranged from partial to total loss over the several experiments performed, which may reflect the transfection efficiency and relative expression of the antisense construct. In the presence of antisense Nell2 only, concentrations of PMA above 300 nM had an affect on I_{Ba} peak amplitude and even this was minimal (data not shown). Taken together, the data suggest that the efficient PKC-dependent modulation of N-type channels requires the presence of Nell2 in HEK cells.

Nell2 and G-protein Dependent Modulation of N-type Calcium Channels

A number of G-protein modulation experiments were carried out in collaboration with Dr. Jhong-Ping Feng (postdoctoral fellow in Dr. Gerald Zamponi's laboratory, University of Calgary). Upon co-expression of Nell2 with N-type channels in HEK cells, there was an approximate 50 % decrease in average current density compared to wild type (**Figure 36**). Application of a strong depolarizing prepulse resulted in a robust increase in peak current activity and a speeding of current kinetics (**Figure 37**). In contrast, prepulses mediated only a minor current enhancement in the absence of Nell2, or upon cotransfection of antisense Nell2.

Prepulse facilitation is a phenomenon observed on N-type calcium channels by G $\beta\gamma$ subunits (Marchetti et al., 1986; Tsunoo et al., 1986, Elmslie et al., 1990; Ikeda, 1991). There was no significant effect of Nell2 on the half inactivation potential (-Nell2: -50.6 $\pm$ 2.8 mV, n=12; +Nell2: -48.3 $\pm$ 3.0 mV, n=9), however, the current voltage relation was shifted by

Figure 34. Time Course of PMA Effect on Co-expressed N-type Channels and Nell2.

N-type calcium channels were co-expressed with Nell2 in HEK cells (108) and current was measured by the perforated whole cell patch clamp technique (0.1 % nystatin) using Ba^{2+} as the charge carrier. Currents were induced by stepping from -100 to +20 mV. The time course was normalized to the current peak amplitude before PMA application and at the indicated time 2.5 nM PMA was applied. In the presence of exogenous Nell2, tenfold decrease in PMA concentration gave the same increase in current amplitude as that observed with N-type channels expressed alone in HEK cells.

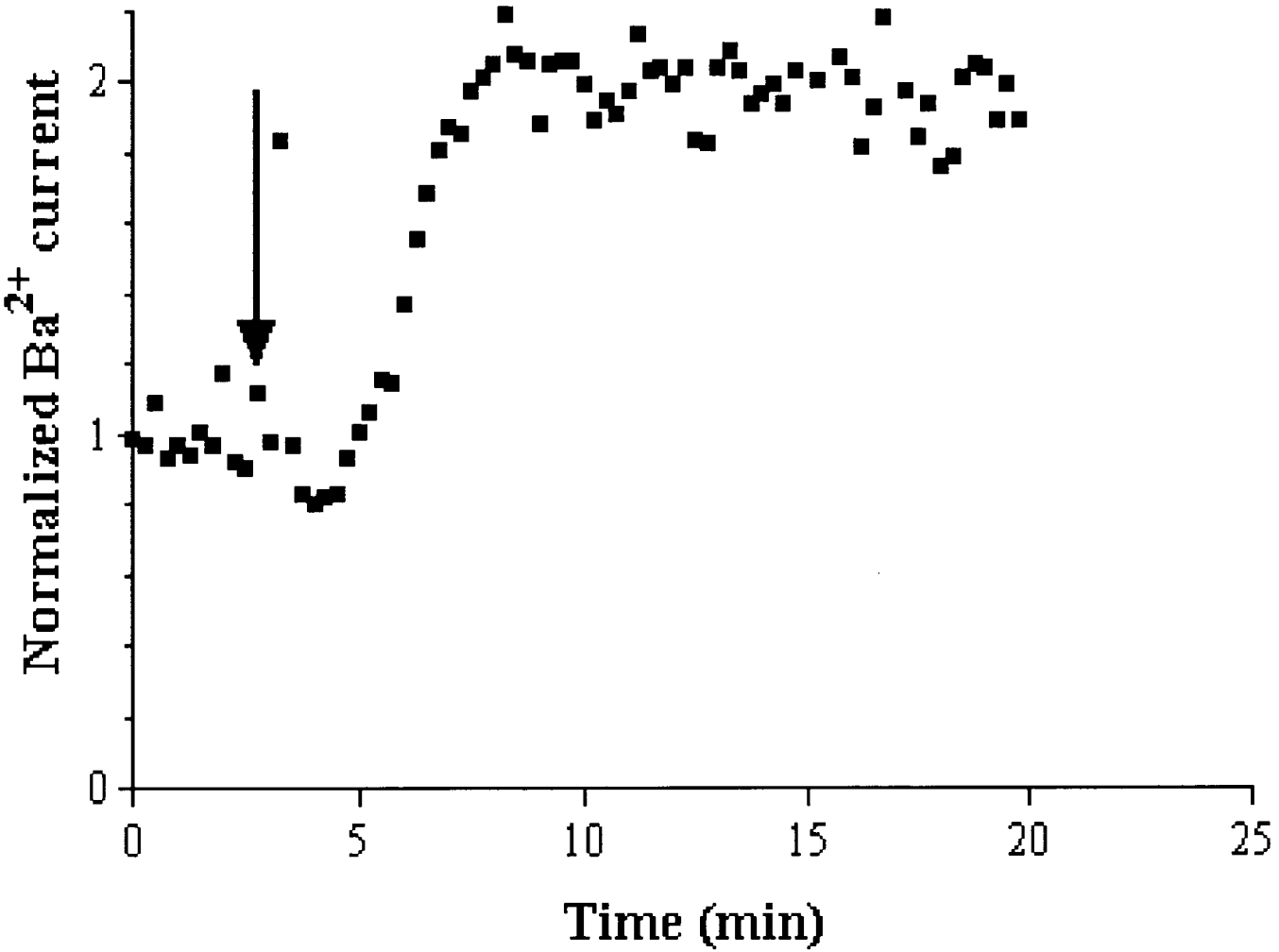
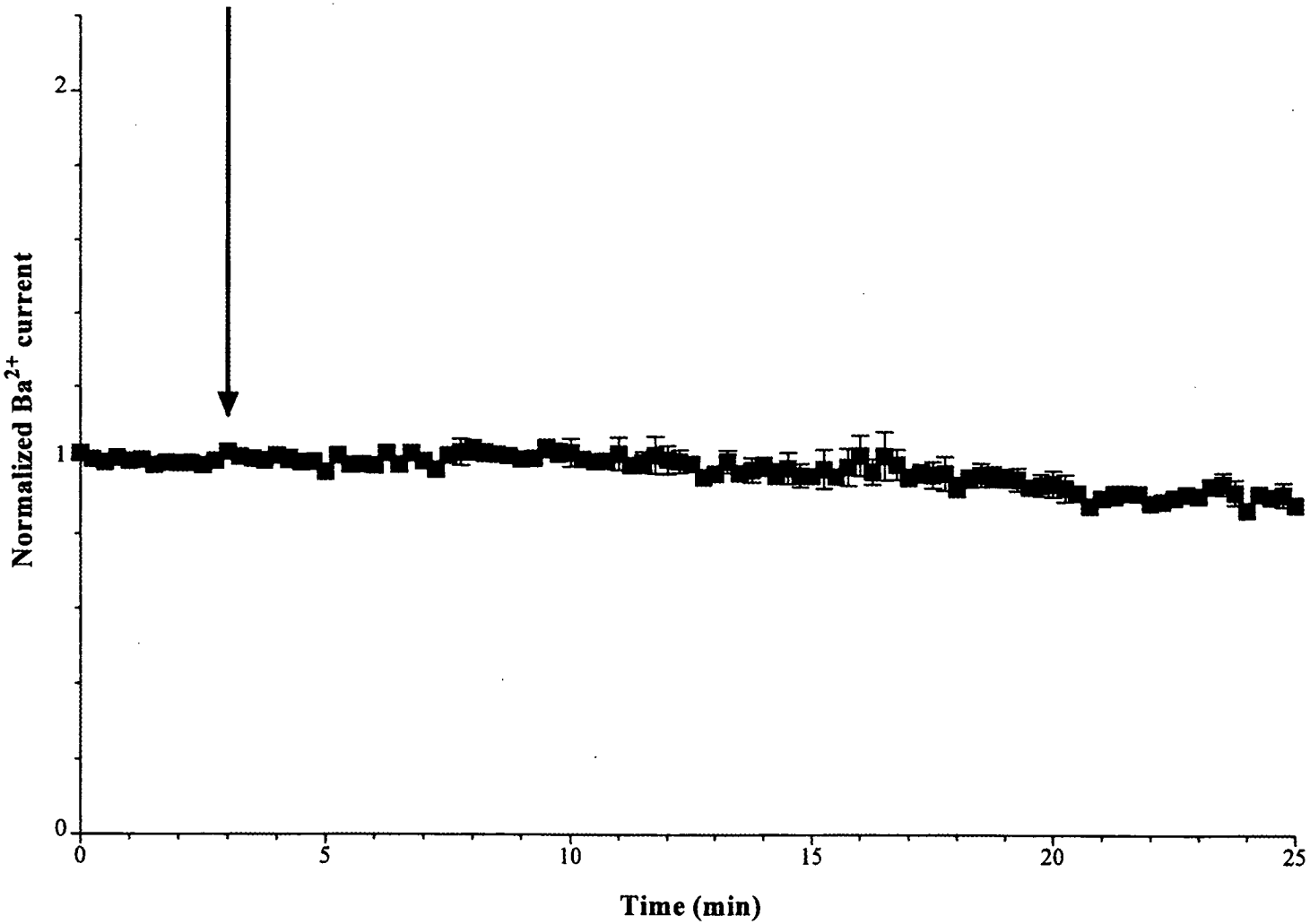

Normalized Ba^{2+} current
0
1
2
0
5
10
15
20
25
Time (min)

Figure 35. Time Course of PMA Effect on Co-expressed N-type Channels and Antisense Nell2.

N-type calcium channels were co-expressed with antisense Nell2 in HEK cells (108) and current was measured by the perforated whole cell patch clamp technique (0.1 % nystatin) using Ba^{2+} as the charge carrier. Cells were stepped from -100 to +20 mV to activate N-type channels. The time course was normalized to the current peak amplitude before PMA application and at the indicated time 25 nM PMA was applied. Co-expression of N-type channels with antisense Nell2 resulted in a loss of increase in current amplitude by PMA, suggesting that Nell2 is required for PKC modulation of N-type calcium channels.

Bath application of 25 nM 4-β-PMA to cells
co-transfected with PKC-BP anti-sense
(Average of n=6 3/98-10/98)



approximately 10 mV in the positive direction upon co-expression with Nell2 (not shown). Both the activation and inactivation kinetics were slowed in the presence of Nell2, in a manner reminiscent of that observed after activation of G-protein coupled receptors. Overall, these data are consistent with a Nell2-mediated tonic inhibition of N-type calcium channels by endogenous G-protein βγ subunits.

To further investigate this possibility, the carboxyl terminal fragment of the β-adrenergic receptor kinase (βARKct, residues 495 to 689, (Magga et al., 2000)) was used as an effective $G_{\beta\gamma}$ sink by binding free $G_{\beta\gamma}$ (Koch et al., 1994). Co-expression of βARKct with Nell2 and N-type channels in HEK cells examined whole-cell currents and found inhibition of peak current when Nell2 was present (with our without βARKct) compared to control N-type channel alone (**Figure 38, top panel**). The ratio of peak whole-cell currents with or without a prepulse resulted in a dramatic decrease in the Nell2 mediated G-protein inhibition with the presence of βARKct (**Figure 38, bottom panel**). The loss of prepulse facilitation with the presence of βARKct indicates Nell2 mediated G-protein enhanced inhibition is acting through the $G_{\beta\gamma}$ complex.

Deletion of the Nell2 binding region on the α_{1B} subunit was expected to prevent the Nell2 mediated G-protein inhibition. Surprisingly, a channel construct lacking the last third of the carboxyl tail region (and thus the putative Bait 4/Nell2 interaction site) displayed a robust tonic G-protein inhibition in the absence of Nell2 (**Figure 38**). The peak currents measured by whole-cell recordings showed a marked reduction in current with the C-terminal deletion of the α_{1B} subunit (**Figure 39, top panel**) and currents were enhanced upon prepulse facilitation compared to wild type (**Figure 39, bottom panel**), suggesting that the carboxyl terminal region of the channel might in fact be inhibitory to G-protein modulation. It is possible that the C-terminus

Figure 36. Reduction of Current Amplitude of N-type Channels Induced by Co-expression of Nell2.

Whole-cell Ba^{2+} currents were recorded from HEK cells expressing N-type channels (α_{1B}, $\alpha_2\delta$ and β_{1b}, WT), with Nell2 (+NELL2) and with antisense Nell2 (+NELL2 antisense). The peak currents were elicited by a test pulse to +20 mV from a holding potential of -100 mV. Peak α_{1B} current is significantly reduced by co-expression with Nell2 but not with antisense Nell2.

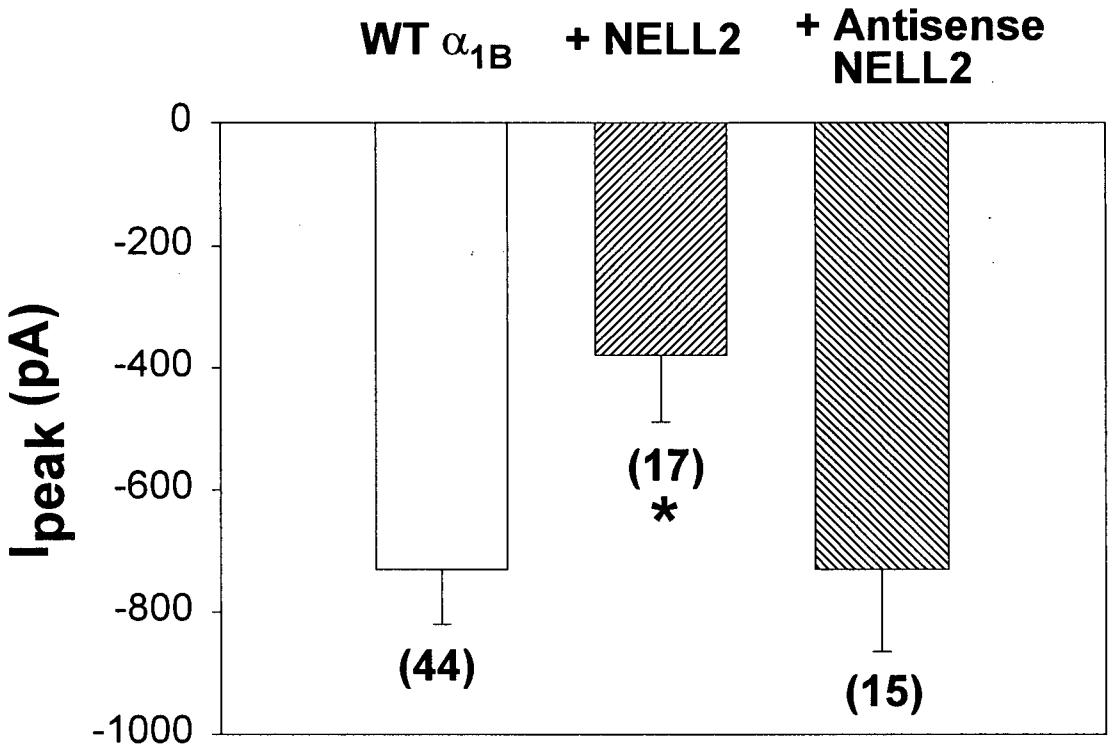

WT α_{1B}
+ NELL2
+ Antisense NELL2
0
-200
-400
-600
-800
-1000
I_{peak} (pA)
(44)
(17)
*
(15)

Figure 37. Nell2 Induced Inhibition of N-type Channels Mediated by Endogenous G-proteins.

Top, representative current traces recorded from the cells expressing N-type channels (WT α_{1B}) alone, co-expressed with Nell2, or with Nell2 antisense. Whole-cell currents were recorded from HEK cells expressing N-type channels with (+PP) or without (-PP) a 50 ms prepulse to +150 mV before a test pulse to +20 mV from a holding potential of -100 mV. The prepulse and test pulse were separated by a 5 ms step return to -100 mV. The Nell2 mediated inhibition of the α_{1B} current is released by the depolarizing prepulse (prepulse facilitation).

Bottom; ratio of the peak currents elicited by the test pulse with or without the depolarizing prepulse was significantly increased by Nell2. In contrast, co-expression with antisense Nell2 did affect either the I_{peak}. Data represent mean ± SEM. The numbers of experiments are indicated in parentheses. * indicates significant difference as comparing to WT α_{1B} in the same figure ($p < 0.05$).

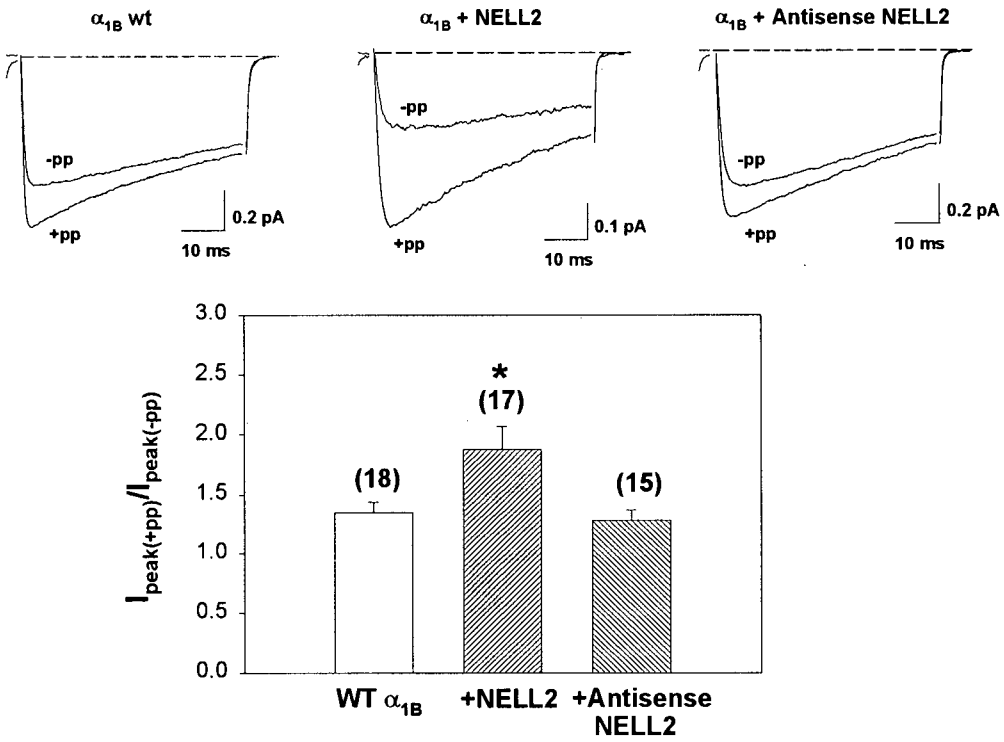

α_{1B} wt
-pp
+pp
0.2 pA
10 ms
α_{1B} + NELL2
-pp
+pp
0.1 pA
10 ms
α_{1B} + Antisense NELL2
-pp
+pp
0.2 pA
10 ms
$I_{peak(+pp)}/I_{peak(-pp)}$
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
(17)
(18)
(15)
WT α_{1B}
+NELL2
+Antisense NELL2

Figure 38. Nell2 Induced Tonic G-protein Inhibition is Diminished by β-adrenergic Receptor Kinase C-terminal Peptide.

Summary of peak whole-cell currents (top) and ratio of peak whole-cell currents (bottom) elicited by a +20 mV test pulse with (+PP) or without (-PP) a +150 mV prepulse recorded from HEK cells expressing WT α_{1B}, $\alpha_2\delta$ and β_{1b} (α_{1B}), with the co-expression of Nell2 and with the co-expression of Nell2 and the β-adrenergic Receptor Kinase C-terminus (βARKct). Nell2 mediated G-protein tonic inhibition was attenuated following expression of βARKct. Data represent mean ± SEM. The numbers of experiments are indicated in parentheses. * indicates significant difference as comparing to WT α_{1B} in the same figure ($p < 0.05$).

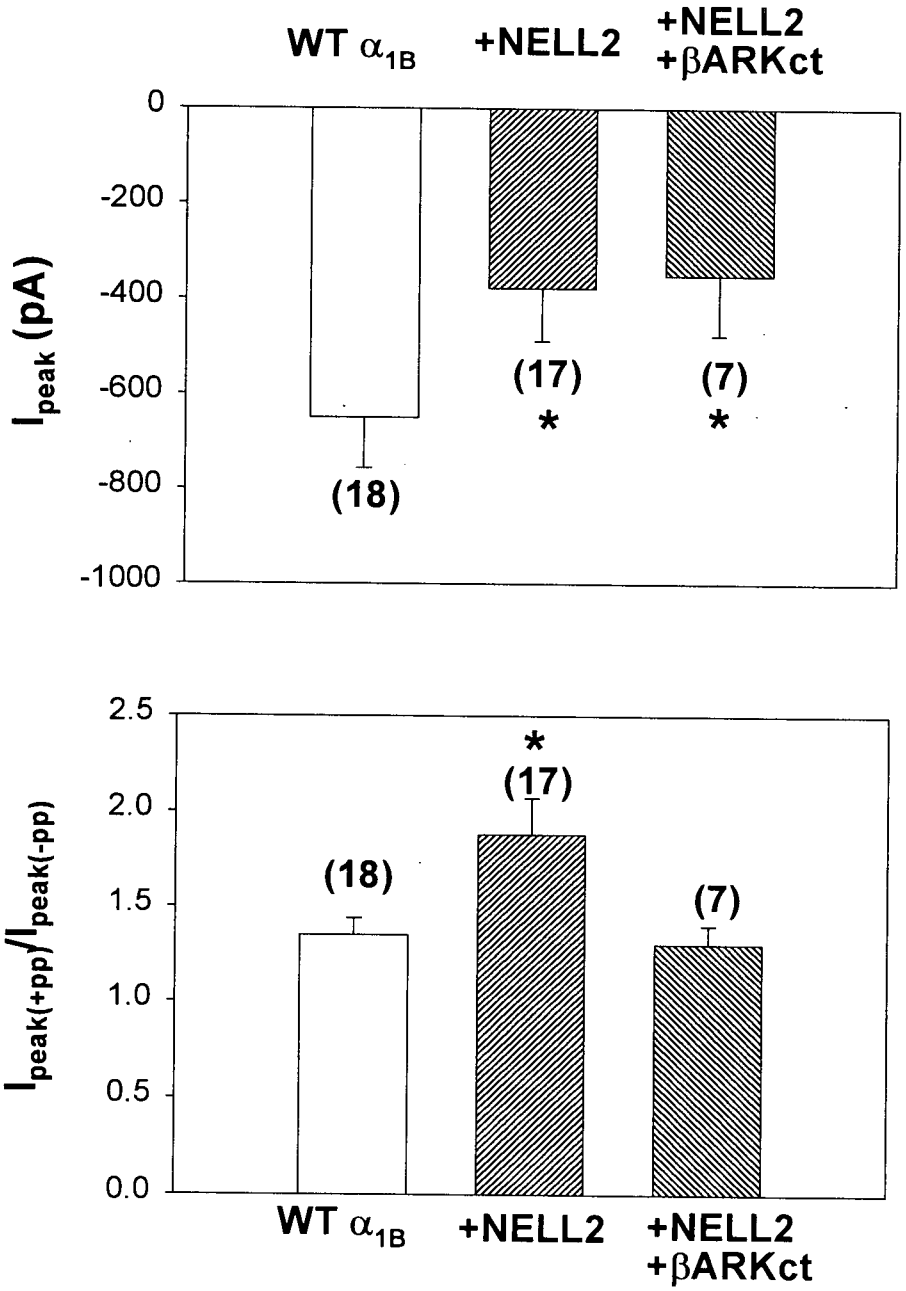
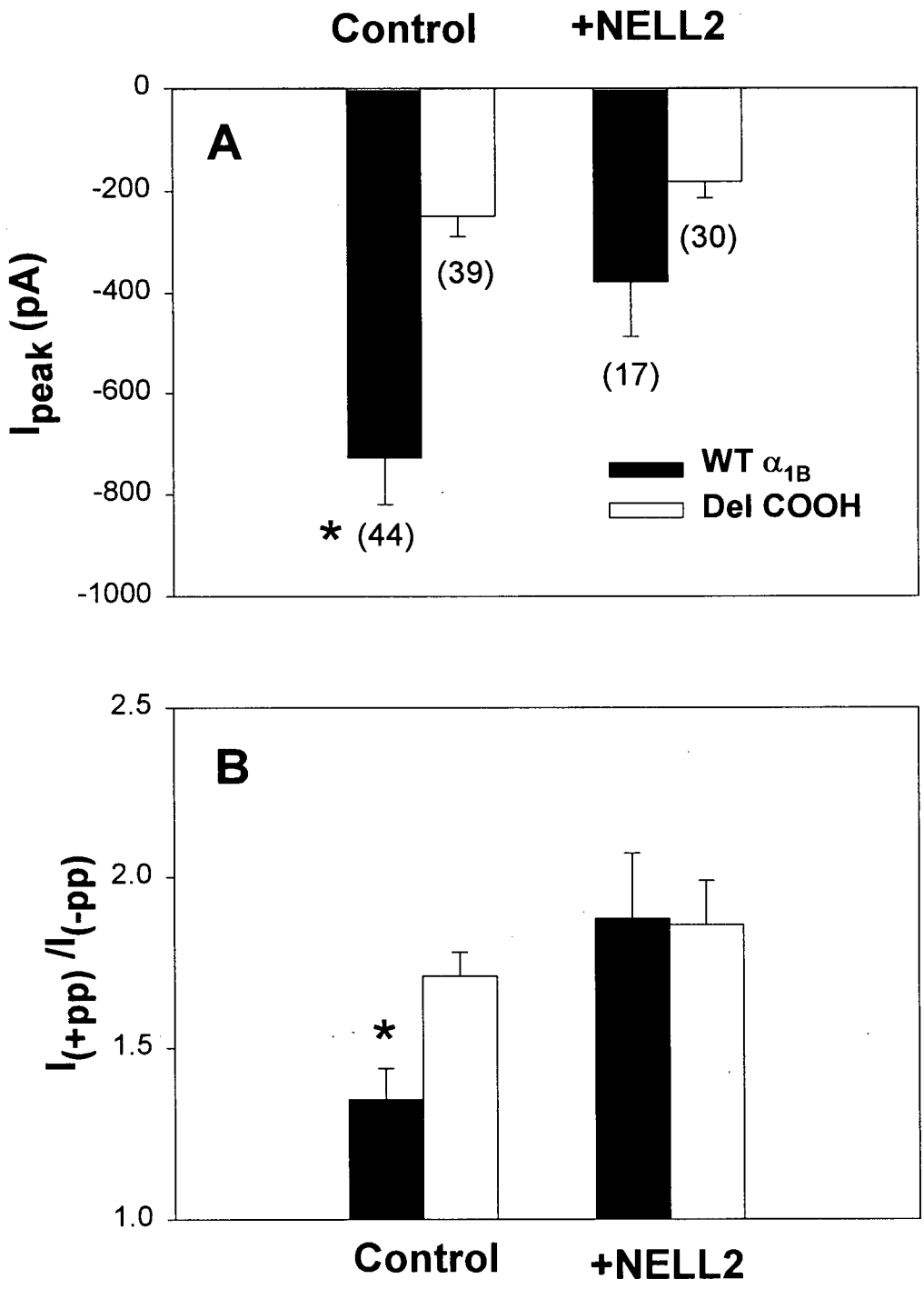

WT α_{1B}
+NELL2
+NELL2
+βARKct
0
-200
-400
-600
-800
-1000
I_{peak} (pA)
(18)
(17)
*
(7)
*
2.5
2.0
1.5
1.0
0.5
0.0
$I_{peak(+pp)}/I_{peak(-pp)}$
(18)
*
(17)
(7)
WT α_{1B}
+NELL2
+NELL2
+βARKct

Figure 39. Nell2 Induced G-protein Inhibition is Mediated via the C-terminus of the α_{1B} Subunit.

Summary of peak whole-cell currents (top) and ratio of peak whole-cell currents (bottom) elicited by a +20 mV test pulse with (+PP) or without (-PP) a +150 mV prepulse recorded from HEK cells expressing WT α_{1B}, $\alpha_2\delta$ and β_{1b} (α_{1B}), or C-terminus deletion mutant of α_{1B} (α_{1B} (del COOH), $\alpha_2\delta$ and β_{1b} subunits). Deletion of the C-terminus of α_{1B} does not seem to effect Nell2 N-channel facilitation. Data represent mean ± SEM. * indicates significant difference as comparing to WT α_{1B} in the same figure ($p < 0.05$).


Control
+NELL2
A
0
-200
-400
-600
-800
-1000
I_{peak} (pA)
(39)
(30)
(17)
* (44)
WT α_{1B}
Del COOH
B
2.5
2.0
1.5
1.0
$I_{(+pp)}$ /$I_{(-pp)}$
*
Control
+NELL2

may act allosterically by hindering other G-protein binding sites (e.g. Zamponi, 1997) and removal of this region enhances G-protein mediated channel inhibition. Alternatively, the C-terminal binding of Nell2 may localize PKC to the channel through the Nell2/PKC complex and removal of the C-terminus attenuates PKC phosphorylation of the channel which favors G-protein-dependent inhibition (Zamponi, 1997).

To test whether the effect of Nell2 was specific to an interaction with N-type channels, or due to a diffuse action on G-protein signaling, over expression of a peptide corresponding to the Bait region of the N-type channel carboxyl tail (N-terminally Flag-tagged Bait 4, fB4) was carried out. **Figure 40** shows that the prepulse effect observed with Nell2 was dramatically attenuated upon over expression of the Bait 4 peptide. In contrast, the Bait 4 peptide did not interfere with G-protein inhibition induced by over expression of exogenous $G_{\beta 1\gamma 2}$, consistent with a mechanism in which the Bait 4 peptide prevents formation of a Nell2-calcium channel complex rather than interfering with G-protein-dependent inhibition in general (**Figure 40, top panel**).

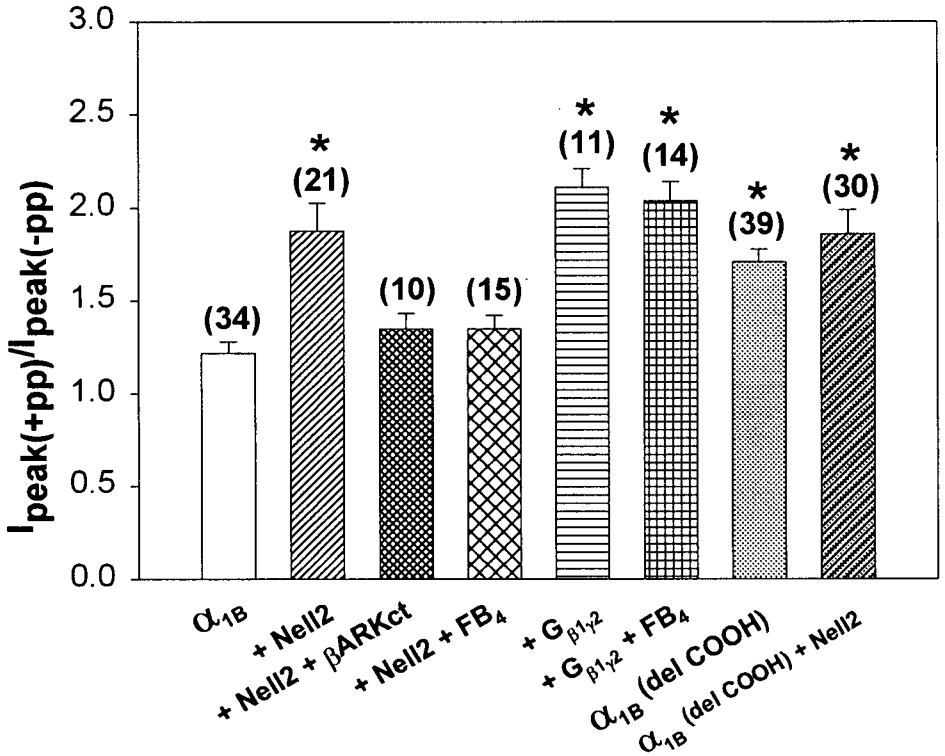
To further investigate the Nell2 structural determinants underlying the G-protein effect, a series of Nell2 deletion mutants were generated and tested for their effects on N-type channel activity (**Table 12**). Deletion of the N-terminal portion of Nell2 abolished the Nell2 induced G-protein inhibition of the channel (**Figure 40, bottom** DMA, -1 to 248 aa). In contrast, deletion of either the middle region (f-DMB, -455 to 649 aa), or the carboxyl terminal portion (f-DMC, -661 to 816 aa) of Nell2 did not result in a loss of the G-protein effect.

Further analysis of this region was performed using deletion mutants of the first 100 amino acids (f-DMD, -1 to 101 aa) and also of the first 20 amino acids (f-DME, -1 to 20 aa) of Nell2 (**Figure 41**). The first 20 amino acids contain a putative signal sequence for Nell2 (**Figure 13**). ^{35}S-

Figure 40. Molecular Determinants of Nell2 Induced G-protein Modulation of N-type Calcium Channels.

Top, summary of ratio of peak whole-cell Ba^{2+} currents elicited by a +20 mV test pulse with (+PP) or without (-PP) a +150 mV prepulse. Recorded from HEK cells expressing WT α_{1B}, $\alpha_2\delta$ and β_{1b} (α_{1B}), or C-terminus deletion mutant of α_{1B} (α_{1B} (del COOH)) co-expressed with Nell2 or Nell2 and β-adrenergic Receptor Kinase C-terminus (βARKct) or Nell2 and Flag-tagged Bait 4 (FB_4) or exogenously expressed $G_{\beta\gamma}$ (+$G_{\beta\gamma}$) or $G_{\beta\gamma}$ and FB_4.

Bottom, summary of ratio of peak whole-cell Ba^{2+} currents elicited as above with WT alone or with co-expression of Nell2 or Flag-tagged Nell2 or Deletion Mutant A or Deletion Mutant B or Deletion Mutant C. Co-expression of WT with Deletion Mutant A (-1 to 248 amino acids of Nell2) attenuated Nell2 mediated G-protein tonic inhibition. Data represent mean ± SEM. The numbers of experiments are indicated in parentheses. * indicates significant difference as comparing to WT α_{1B} in the same figure ($p < 0.05$).


3.0
2.5
2.0
1.5
1.0
0.5
0.0
$I_{peak(+pp)}/I_{peak(-pp)}$
(34)
*
(21)
(10)
(15)
*
(11)
*
(14)
*
(39)
*
(30)
α_{1B}
+ Nell2
+ Nell2 + βARKct
+ Nell2 + FB_4
+ $G_{\beta 1\gamma 2}$
+ $G_{\beta 1\gamma 2}$ + FB_4
α_{1B} (del COOH)
α_{1B} (del COOH) + Nell2

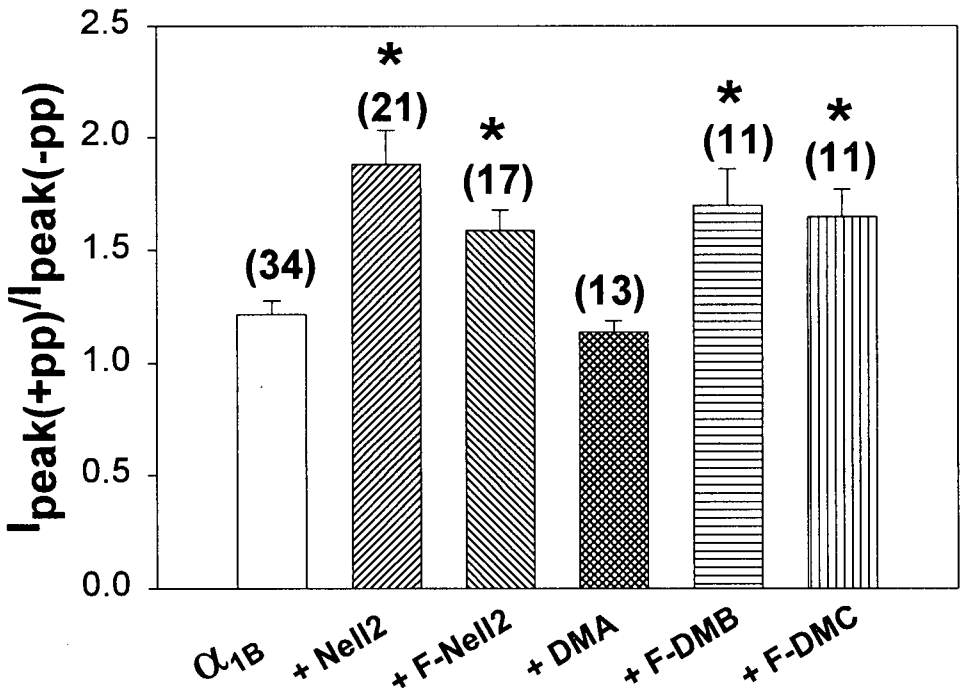

2.5
2.0
1.5
1.0
0.5
0.0
$I_{peak(+pp)}/I_{peak(-pp)}$
(34)
*
(21)
*
(17)
(13)
*
(11)
*
(11)
α_{1B}
+ Nell2
+ F-Nell2
+ DMA
+ F-DMB
+ F-DMC

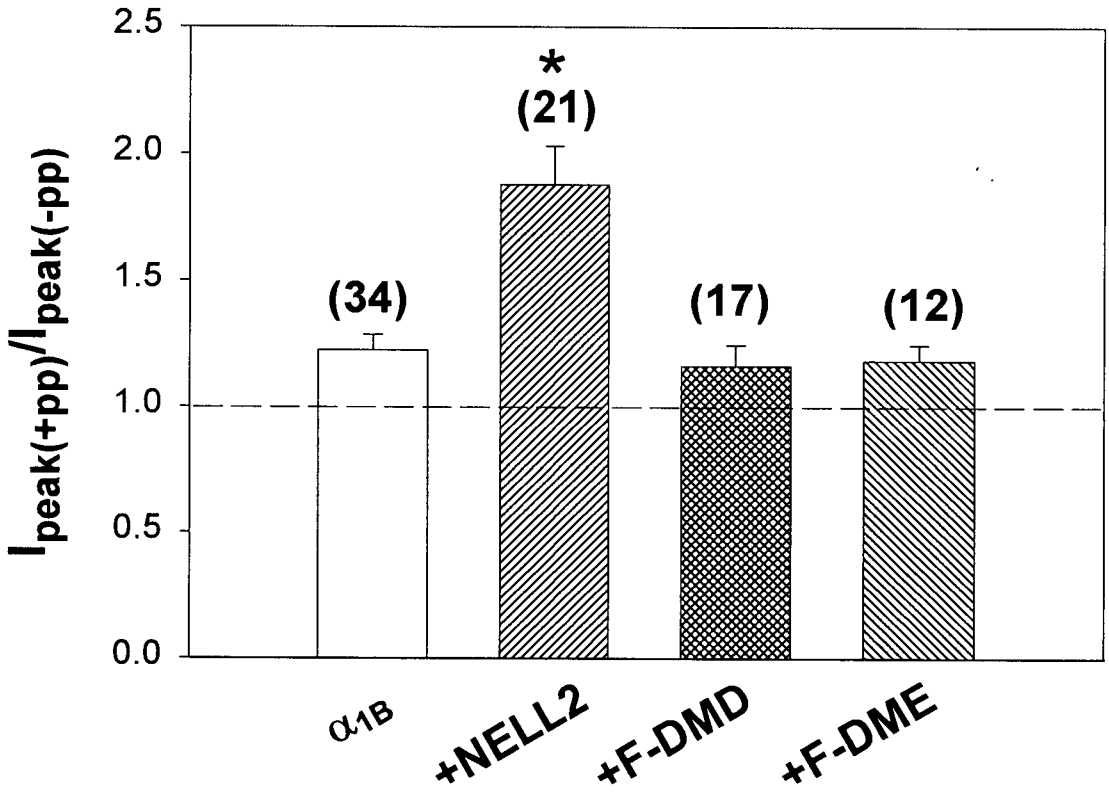
Table 12. Nell2 Deletion Mutants Tested for Nell2 Mediated G-protein Inhibition of N-type Calcium Channels.

Regions of Nell2 were deleted as described in Materials and Methods. Nell2 Deletion Mutants B, C, D and E were N-terminally Flag-tagged. Deletion Mutants C, D and E were *in vitro* translated and shown to be the correct molecular size (**Figure 42**). All Nell2 deletion mutant constructs were co-expressed in HEK cells with N-type channels and tested for loss of Nell2 mediated G-protein inhibition and the Nell2 N-terminus was found to be essential. The central (Deletion Mutant B) and C-terminus (Deletion Mutant C) regions of Nell2 were not required.

Nell2 Deletion Mutant	Flag-tagged Epitope	Nell2 Residues Deleted	Loss of Nell2 Mediated G-protein Inhibition
Deletion Mutant A (DMA)	-	-1 to 248	+
Deletion Mutant B (f-DMB)	+	-455 to 649	-
Deletion Mutant C (f-DMC)	+	-661 to 816	-
Deletion Mutant D (f-DMD)	+	-1 to 101	+
Deletion Mutant E (f-DME)	+	-1 to 20	+

Figure 41. Interaction Site Between N-terminus of Nell2 and the N-type α_{1B} Subunit.

Summary of ratio of peak whole-cell Ba^{2+} currents elicited by a +20 mV test pulse with (+PP) or without (-PP) a +150 mV prepulse recorded from HEK cells expressing WT α_{1B}, $\alpha_2\delta$ and β_{1b} (α_{1B}) alone or co-expressed with Nell2 or Deletion Mutant D (F-DMD) or Deletion Mutant E (F-DME). Both co-expression of Deletion Mutants D or E attenuated Nell2 mediated G-protein tonic inhibition. Data represent mean ± SEM. The numbers of experiments are indicated in parentheses. * indicates significant difference as comparing to WT α_{1B} in the same figure ($p < 0.05$).


2.5
2.0
1.5
1.0
0.5
0.0
$I_{peak(+pp)}/I_{peak(-pp)}$
*
(21)
(34)
(17)
(12)
α1B
+NELL2
+F-DMD
+F-DME

methionine labeled *in vitro* translation deletion mutants C, D and E exhibited the predicted molecular sizes (**Figure 42**). Deletion mutant E was transfected into HEK cells and membrane staining appeared identical to that of the full length Nell2 (**Figure 43**) suggesting cell processing was not a factor in loss of protein function. **Figure 44** summarizes the data and suggests that the N-terminal portion of Nell2 may comprise the minimal domain required for G-protein enhanced inhibition of the N-type channel.

Figure 42. *In vitro* Translated Nell2 Deletion Mutants C, D and E.

Autoradiogram of *in vitro* translated Flag-tagged Nell2 (Flag/Nell2, 92 kDa), Flag-tagged Deletion Mutant C (Flag/Del Mut C), Flag-tagged Deletion Mutant D (Flag/Del Mut D), Flag-tagged Deletion Mutant E (Flag/Del Mut E) and pGemTE vector (Negative Control). See **Figure 44** and **Table 12** of G-protein Functional Analysis Results for Deletion Mutant construct information.

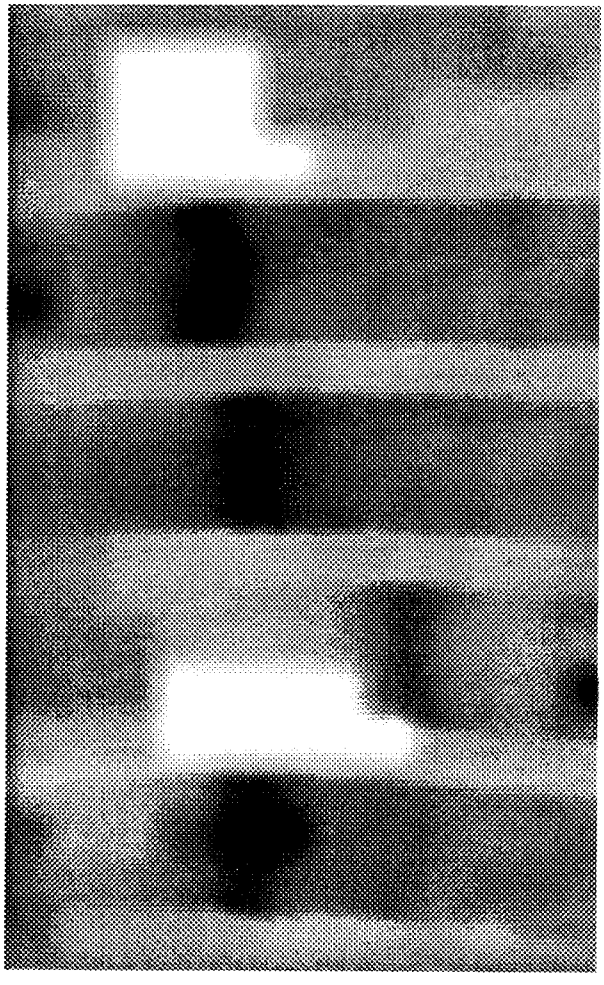

Negative Control
Flag/Del Mut E
Flag/Del Mut D
Flag/Del Mut C
Flag/Nell2
100 -
71 -

Figure 43. HEK Cell Expressed Nell2 Flag/Deletion Mutant E.

HEK 201 cells were transiently transfected with carrier DNA (pGemTE vector, Untransfected) or Flag-tagged Deletion Mutant E (Flag/Del Mut E). Twenty-four hours post transfection, cells were fixed and stained with primary mouse monoclonal M5 (anti-Flag) antibody and secondary anti-mouse conjugated to Texas Red. DAPI staining indicates the cell nucleus. Deletion Mutant E (-1 to 20 amino acids of Nell2) shows the same staining patterns as Nell2 in HEK 201 cells.

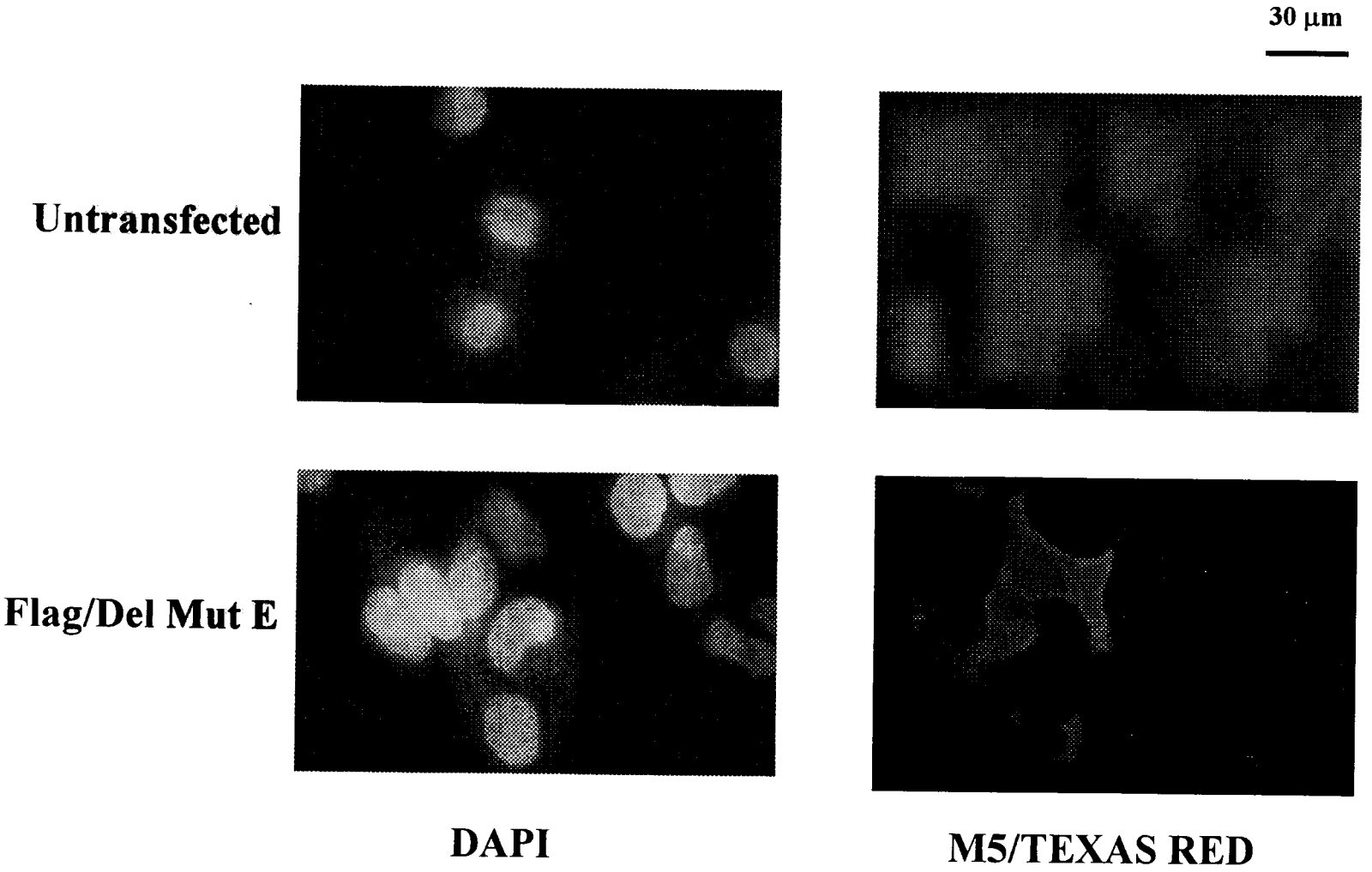
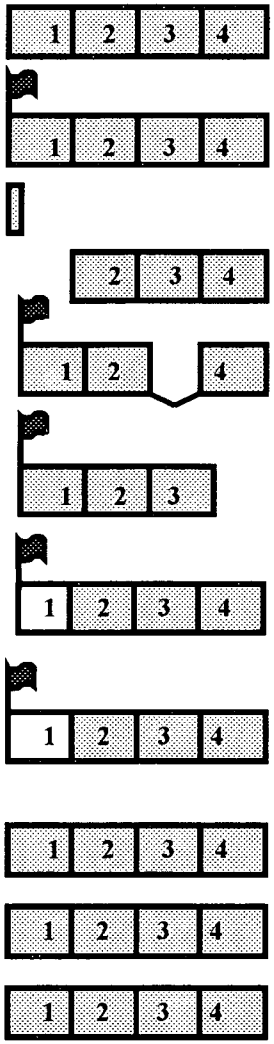

30 µm
Untransfected
Flag/Del Mut E
DAPI
M5/TEXAS RED

Figure 44. Summary of G-protein Functional Analysis.

Data summarizing results from experiments where whole-cell currents elicited by a +20 mV test pulse with (+PP) or without (-PP) a +150 mV prepulse recorded from HEK cells expressing various proteins. An enhanced G-protein response indicates those constructs which had an increase in current amplitude after a prepulse compared to no prepulse. N-type channels are the co-transfection of α_{1B}, $\alpha_2\delta$ and β_{1b} subunits. The boxes (1-4) represent approximately 200 amino acid regions of Nell2. The flag indicates those clones that were N-terminally tagged with the Flag epitope.



Transfected HEK Cells	Enhanced G Protein Response
Nell2 + N–type	+
Flag/Nell2 + N–type	+
Antisense Nell2 + N–type	-
Deletion Mutant A (-1 to 248 aa) + N–type	-
Flag/Deletion Mutant B (-455 to 649 aa) + N–type	+
Flag/Deletion Mutant C (–661 to 817 aa) + N–type	+
Flag/Deletion Mutant D (-1 to 101 aa) + N–type	-
Flag/Deletion Mutant E (-1 to 20 aa) + N–type	-
Nell2 + N–type + βARKct	-
Nell2 + α1Bc-term del β1b α2δ	+
Nell2 + N–type + Flag/Bait 4	-

Chapter 6. Discussion

Sequence Comparison of Nell with Others

To date, two different Nel isoforms (Nell1 and Nell2) have been described in several species including chick, rat, mouse and human (Bading et al., 1993, Matsuhashi et al., 1995, Watanabe et al., 1996, Beckmann et al., 1998, Kuroda and Tanizawa, 1999, Luce and Burrows, 1999). The rat Nell2 identified in this thesis by interaction with the N-type calcium channel has been previously characterized by other groups (Kuroda et al., 1999, Kuroda and Tanizawa, 1999, Luce and Burrows, 1999, Oyasu et al., 2000). There are some differences between the clone described here and the previously published Nell2 clone. Comparatively, Kuroda's rat Nell2 sequence (NP_112332) contains ten nucleotide differences which result in seven amino acid changes (**Table 13**). Interestingly, both clones were generated by PCR amplification of rat brain RNA (Kuroda et al., 1999, Kuroda and Tanizawa, 1999, Guthrie, 2001) and Kuroda's Nell2 (NP_112332) was also isolated from the yeast two-hybrid library from Clontech, the same library used in my research.

Although the initial clones isolated in the yeast two-hybrid screens did not contain the entire coding region, a full-length rat Nell2 was amplified using 5' oligonucleotides from the previously published N-terminal sequence (Kuroda et al., 1999, Kuroda and Tanizawa, 1999) and the yeast two-hybrid clone sequences. Only two amino acid changes were observed between the full-

Table 13. Sequence Comparison of the Rat Nell2 Clones.

Comparison of the previously cloned rat Nell2 (NP_112332, Kuroda et al., 1999; Kuroda and Tanizawa, 1999) and the Nell2 clone discussed in this thesis have seven amino acid changes. The amino acid change refers to the previous clone and the clone discussed here. Some of these changes lie within regions predicted to be important, including the putative binding region between Nell2 and the N-type α_{1B} subunit. When compared to the isolated yeast two-hybrid clones (which were also amplified by PCR as a Clontech library from rat brain), only two changes of the seven did not match (*). Not applicable (-). 1. The yeast two-hybrid clones did not overlap the N-terminal sequence of Nell2.

Residue	Amino Acid Change	Domain	Yeast Two-hybrid Sequences
49	P Δ S	-	1
101	G Δ E	-	1
408	M Δ T	-	M*
532	G Δ E	Putative Binding Region to α_{1B}, Motif Common to G-protein Coupled Receptors	G*
673	Q Δ R	EGF-like Domain	R
716	A Δ V	-	V
727	E Δ G	-	G

length PCR-generated clone and the yeast two-hybrid partial clones. Therefore, of the seven amino acid differences observed between the two rat Nell2 full-length clones, three of the changes appear to be conserved, two do not overlap with the yeast two hybrid partial clones and two can be considered PCR errors. Errors in sequence can be introduced by PCR and have been attributed to several factors including pH, concentrations of deoxynucleoside triphosphates, magnesium ion, Taq polymerase and template secondary structure (Ling et al., 1991; Loewen and Switala, 1995).

It should be noted that the amino acid change at position 532 is within the Nell2 putative binding region to the C-terminus of the α_{1B} subunit. Several attempts at demonstrating direct binding between Nell2 and the C-terminus of the α_{1B} subunit were performed (Chapter 4) and it is possible that the change from an uncharged glycine to the negatively charged glutamate residue may have contributed to the lack of binding in the experiments conducted. This could be tested in the future by utilizing *in vitro* mutagenesis to engineer the change back to a glycine.

RNA Distribution of Nell2 and the N-type Channel

Using RT-PCR and Southern blot techniques, Nell2 and N-type channel mRNA distributions were examined in various rat tissues. N-type channel α_{1B} subunit mRNA was thought to be exclusively expressed in the nervous system (Coppola et al., 1994; Dubel et al., 1992). Nell2 mRNA expression was previously reported as being localized to the nervous system and kidney in adult, while ubiquitously expressed in all tissues during development (Matsuhashi et al., 1995; Matsuhashi et al., 1996; Watanabe et al., 1996). Here Nell2 and the α_{1B} subunit expression were both found in rat brain stem, cerebellum, cortex and pituitary with trace amounts in eye and heart. There were also some tissue distribution differences as N-type channels were found in liver and kidney, while Nell2 was not. Because N-type channel mRNA was not only found in

tissues expressing Nell2 mRNA, these proteins may not always be co-expressed or associated. It is possible that Nell2 may have a number of physiological functions and interact with other cellular proteins.

Subcellular Distribution of Nell2

The subcellular distribution of Nell2 was analyzed to determine whether Nell2 co-localized with N-type channels in HEK cells and neurons. The N-type α_{1B} subunit has previously been shown to localize to plasma membranes in both HEK cells and neurons (Dubel et al., 1992; Westenbroek, 1992, Williams et al., 1992, Fujita et al., 1993, Witcher, 1993, De Waard, 1994). Based on sequence analysis, the rat Nell2 clone contains a signal sequence and a cleavage site. These features are common in secreted proteins although some proteins contain a signal sequence and are not secreted. The packaging events end at the membrane and the protein becomes membrane associated. In the case of Kuroda, a Flag epitope tag was added to the N-terminus of Nell2 and expression was analyzed in COS-7 cells. No expression was detected and it was hypothesized that the N-terminal tag disrupted the signal sequence. The Flag epitope tag was then placed on the C-terminus of Nell2 and expression was described in COS-7 cells. Surprisingly, even though both Kuroda's and the Nell2 isolated here are very similar, expression patterns differed between Kuroda's C-terminally Flag-tagged Nell2 in COS-7 cells and the work presented here for HEK cells.

In the present study, an N-terminal Flag epitope tag was added to rat Nell2 and protein expression was detected in HEK cells by Western blot. The data show that Nell2 is predominantly in the membrane fraction and not found in HEK cell extract. Kuroda's C-terminal Flag-tagged Nell2 expression was detected by Western blot as a 140 kDa protein in the cytoplasm, membrane and tissue culture medium of COS-7 cells (Kuroda et al., 1999). When

protein isolates were treated with N-glycanase, the 140 kDa band was reduced to 90 kDa, suggesting glycosylation events were responsible for the increase in molecular weight (Kuroda et al., 1999). The work presented in this thesis suggests Nell2 is membrane associated and co-localizes to the same region as N-type channels in HEK cells. This was not only demonstrated by Western blot (**Figures 25 and 26**), but also by immunohistochemistry (**Figures 22 to 24**). Nell2 staining was also demonstrated in membranes in rat cultured primary cortical neurons and found to be similar to that of the N-type channel (**Figure 27**). Given the distinct molecular size of Nell2 in COS-7 and HEK cells, the difference between the Nell2 subcellular distribution reported here and those of Kuroda are likely due to the different cell types used for exogenous expression.

Structural Aspects of Nell2 Primary Sequence

The protein sequence of Rat Nell2 was analyzed for known consensus structural and functional domains and found to contain a signal sequence with a signal peptide cleavage site, twelve potential PKC phosphorylation sites, eight EGF-like repeat domains (three overlap), seven N-glycosylation sites, three casein kinase II phosphorylation sites, two tyrosine kinase phosphorylation sites, twelve N-myristoylation sites, three aspartic acid and asparagine hydroxylation sites and two VWFC domain signatures. Nell2 sequence also contains two motifs common to G-protein coupled receptors. The putative α_{1B} subunit binding site includes one of the common G-protein coupled receptor motifs as well as two EGF-like repeats, one of which has calcium binding potential.

The most predominant protein motif of Nell2 is the five EGF-like repeats. EGF-like repeats are generally found in the extracellular domain of membrane-bound proteins or in proteins known to be secreted, although some exceptions exist (e.g. prostaglandin G/H synthase, Picot et al., 1994). EGF-like domains function in protein-protein interactions including cell coagulation and

proliferation, cell growth and differentiation (Carpenter and Zendegui, 1986, Davis, 1990, Boonstra et al., 1995). The Nel family of proteins contain five to six EGF-like repeat domains and the rat Nell2 EGF-like repeats have been shown to bind specific PKC isoforms (Kuroda and Tanizawa, 1999). A BLAST search of Nell2 putative binding region to the α_{1B} subunit resulted in the identification of the Nel family members, fibrillin and notch family members. The common feature of all three groups of proteins is the EGF-like repeats. Although these proteins contained EGF-like repeats, these domains were the only commonality and the complete putative binding region seems to be unique to Nell2.

Some EGF-like repeats are calcium binding and are important in protein conformation. Point mutations in the calcium binding residues can render some proteins containing these domains biologically inactive and can lead to diseases such as hemophilia B (mutation in factor IX), Marfan syndrome (mutation in fibrillin) and hypercholesterolemia (mutation in low density lipoprotein receptor) (Stenflo et al., 2000). A calcium binding EGF-like domain also exists within the Nell2/α_{1B} subunit putative binding region and may regulate the interaction through calcium concentration.

Another interesting domain of Nell2 is the metabotropic G-protein coupled receptor domain. This consensus sequence is found in most of the metabotropic glutamate receptors (mGluRI-VIII) and are known as G-protein coupled receptor family 3 proteins. The cysteine rich regions of these domains are crutial for protein folding and ligand binding (Peltekova et al., 2000). Cysteine residues not only stabilize protein conformation but also contribute to protein-protein interactions. One of these cysteine rich domains (containing nine cysteine residues) is located in the putative binding region between Nell2 and the α_{1B} subunit.

The Nell2 potential phosphorylation sites are also an interesting finding since other proteins that bind PKC have been known to be regulated by phosphorylation (Mochly-Rosen, 1995). The anchoring of proteins can increase regulation of kinases and phosphorylation events can contribute to further control mechanisms. Some protein interactions with PKC are affected by phosphorylation, while others are not. For example, both non-phosphorylated and phosphorylated AKAP79 binds PKC (Klauck et al., 1996). Also, the binding of MAP2B to the α_{1C} subunit has been demonstrated with and without phosphorylation of α_{1C} (Davare et al., 1999). In contrast, the RII region of PKA, which binds AKAPs, has been shown to disrupt binding to MAP2B by phosphorylation of threonine 69 (Keryer et al., 1993). Thus, it is possible that Nell2 may be regulated by phosphorylation itself and provide another mechanism of control for N-type modulation.

Functional Considerations of Nell2

Binding to PKC

To date, eleven PKC isoforms exist and are grouped according to their N-terminal structures (Liu and Heckman, 1998). The typical class includes the α, β (I and II) and γ; the novel class includes δ, ε, η, θ and μ; and the atypical class includes the isoforms ζ and ι/λ. The typical PKC isoforms are regulated by calcium, phorbol esters or diacylglycerol while the novel PKC isoforms are calcium independent but still diacylglycerol-stimulatable. The atypical isoforms do not bind phorbol esters and are calcium and phosphodiesterase independent. The atypical PKC isoforms are regulated by lipid cofactors such as phosphatidylinositol (3,4,5)trisphosphate and ceramide. Functionally, the novel and atypical PKCs are involved in cell proliferation. Brain tissues express all known PKC isoforms (Huang and Huang, 1993; Liu and Heckman, 1998).

The yeast two-hybrid system has proven to be a common way to identify proteins which bind

PKC. Rat Nell1 and Nell2 proteins were initially identified using the yeast two-hybrid system with PKCβI regulatory region as the Bait (Kuroda et al., 1999). Those proteins identified as PKC binding-proteins are proposed to function as anchor proteins, although with the case of the Nell1 and Nell2 proteins, the substrates for PKC are yet to be determined. Anchor proteins are believed to localize kinases and phosphatases to subcellular targets, limit access to substrates and ultimately to play a role in signal transduction so that specificity can be maintained within specific sites of a cell (Dodge and Scott, 2000).

Differential binding to PKC regulatory domains of different isoforms has been observed between the Nell1 and Nell2 isoforms. Nell1 exhibits affinity binding to PKCδ, moderate affinity for PKCβI and ζ and no binding with α, γ or ε isoforms. In contrast, Nell2 was found to bind with high affinity to PKCβI, ε and ζ and with lower affinity to γ and δ. Interestingly, neither Nell1 nor Nell2 showed binding with the PKC α isoform (Kuroda and Tanizawa, 1999).

The EGF-like domains of Nell2 have been demonstrated as the region which binds PKC (Kuroda and Tanizawa, 1999), yet are not found in other PKC binding-proteins. EGF-like repeats are regions of 50 to 60 amino acid residues with six highly conserved cysteine residues. Another PKC binding-protein, RBCC protein interacting with PKC 1 (RBCK1), contains three cysteine-rich regions (Tokunaga et al., 1998) and thus cysteine residues may generally be important in PKC binding domains. The identification of the N-type channel association to a protein which binds PKC is significant in that modulation by PKC has been well established and now a mechanism for regulation can be investigated.

Nell2 as an Anchoring Protein

The exact functions of the Nel protein family has yet to be elucidated, but based on their similarity to known EGF family members, Nels are thought to play a role as signaling molecules, contributing to cell growth, development and oncogenesis (Pierga and Magdelenat, 1994, Ma and Calvo, 1996, Kuroda and Tanizawa, 1999, Luce and Burrows, 1999). However, it is possible that the Nell2 EGF-like domains have an alternate role to the previously characterized developmental functions and may provide a mechanism to anchor PKC to the N-type calcium channel and other possible targets.

The examination of scaffold and anchoring-proteins has shed light onto the complexity of the molecular organization at synapses and have also helped to define the mechanisms that underlie channel regulation by targeting kinases and phosphatases and enhancing specificity at sites of action (Dodge and Scott, 2000; Fraser and Scott, 1999). Nell2 can now be considered an anchoring-protein, localizing PKC to the N-type channel for fast and efficient phosphorylation and resulting in the rapid modulation of neurotransmission at synapses. Nell2 may also contribute to the structural stability of the channel by binding PKC or allosterically regulating the channel to favor the activity of PKC or G-protein actions. Nell2 binds a region of the α_{1B} subunit C-terminus in the yeast two-hybrid system, but other binding sites may also exist. The $G_{\beta\gamma}$ subunit and calcium channel β subunit are thought to have multiple binding sites on the α_1 subunit (Pragnell et al., 1994, Zhang et al., 1996, De Waard et al., 1997, Herlitze et al., 1997).

It is also possible that Nell2 binds additional proteins including other ion channels. The channel-associated proteins of synapses (chapsyns) include several family members (PSD-95/SAP90, SAP97/hdlg, chapsyn110/PSD-93 and SAP102) and interact with both the shaker potassium channels and the NR2 subunits of NMDA receptors (Sheng and Kim, 1996). Anchoring-proteins that bind PKC have also been shown to bind several different ion channels. For example,

AKAP15/18 and AKAP79 have been linked to several ion channels (Fraser and Scott, 1999). Finally, it is also possible that Nell2 binds more than one enzyme since AKAP79 acts as a scaffold protein for three signaling enzymes (PKA, CaN and PKC, Klauck et al., 1996).

Significant complexity exists *in vivo* and there are likely multiple large protein complexes within the synaptic terminal. Although Nell2 anchors PKC to the channel, the α_{1B} subunit is likely not the only target of PKC phosphorylation. For example, it has been demonstrated that activation of the PKC pathway can modulate N-type channels (Yang and Tsien, 1993) as well as proteins that interact with N-type channels such as syntaxin (Yokoyama et al., 1997). It is also possible that the auxiliary calcium channel subunits can be modulated by PKC as well. For example, it has been demonstrated in *Xenopus* oocytes that L-type currents are modulated by activation of the PKC pathway and require the presence of the β_1 subunit (Bouron et al., 1995), although it is the α_1 subunit that is preferentially phosphorylated (Chang et al., 1991).

It appears that syntaxin-1A and Nell2 have similar functions in modulation of N-type channels; both enhance G-protein inhibition and both act to anchor proteins to the channel. Syntaxin-1A binds both the N-type α_{1B} subunit and the $G_{\beta\gamma}$ subunit and is hypothesized to localize the $G_{\beta\gamma}$ subunit to its calcium channel binding sites for fast and efficient channel inhibition (Jarvis et al., 2000; Lu et al., 2001). Since PKC phosphorylation of the α_{1B} subunit attenuates $G_{\beta\gamma}$ subunit binding, syntaxin-1A and PKC are thought to be antagonistic regulators of N-type inhibition (Lu et al., 2001).

Nell2 may enhance N-type channel inhibition by attenuating PKC phosphorylation,

particularly at the $G_{\beta\gamma}$ binding site, by binding PKC and rendering the enzyme inactive. Perhaps another important role for Nell2 may be to inhibit PKC phosphorylation of the Synprint site by binding PKC and thereby allowing syntaxin to bind, localizing the $G_{\beta\gamma}$ subunit and favoring the G-protein pathway of modulation. Although this may occur *in vivo*, an enhanced G-protein modulation was present upon co-expression of Nell2 alone (HEK cells do not express syntaxin-1A endogenously, Jarvis and Zamponi, 2001) and the affects were inhibited by Bait 4 peptide, supporting the fact Nell2 interaction with the C-terminus of the α_{1B} subunit is involved in the G-protein effect without involvement of syntaxin-1A.

The concept of localizing G-proteins to their downstream effector molecules is not new. As reviewed by Laporte et al. (2001), intracellular signaling is a selective, rapid process which is controlled by organizing macromolecular complexes at the plasma membrane. This was nicely demonstrated by (Davare et al., 2001) where a direct interaction between the G-protein coupled receptor β_2 adrenergic receptor and the L-type α_{1C} subunit was described. The model postulated a multiprotein complex with intrinsic signaling which included effectors such as G-proteins, adenylyl cyclase, cAMP-dependent kinase (PKA) and phosphatase (PP2A). The complex is further organized by the AKAP microtubule-associated protein MAP2B which recruits PKA to the α_{1C} subunit resulting in upregulation of channel activity (Davare et al., 1999).

Nell2 may be a unique anchor protein, the first identified to contain EGF-like repeats which are not only involved in binding PKC but also in binding to an ion channel (Kuroda and Tanizawa, 1999, Guthrie, 2001). PKC isozymes have both distinct cellular functions and differential localization within a cell (Mochly-Rosen, 1995). Anchoring PKC is critical within a neuron because this enzyme is present in the cytosol and activators are present at the membrane

(Nishizuka, 1992) and the translocation process takes time. Neurotransmission events occur within milliseconds and the fast and rapid execution of vesicle release is required. Coordination of multiple proteins within presynaptic terminals regulated by space and time results in efficient neurotransmitter release and identifying Nell2 as an anchor protein helps in understanding the molecular basis of the N-type channel contributions to this process.

N-type/Nell2 Interaction

The full-length Nell2 and the yeast two-hybrid clones consisting of partial fragments of Nell2 were incubated with the C-terminus of the α_{1B} subunit under various conditions and standard co-immunoprecipitation experiments were carried out (Harlow, 1988). Proteins were expressed in E. coli, HEK cells and in-vitro translated with epitope tags such as GST, myc, HIS and Flag. Proteins were isolated using standard beads such as protein A and protein G as well as commercially available coupled beads like Anti-flag M2-agarose Affinity Gel (Sigma). Rabbit generated antibodies (anti-Nell2), mouse generated antibodies (Anti-Myc, Wilf Jefferies laboratory) and commercially available antibodies were used for these experiments (see Chapter 2).

Many experiments were carried out in attempt to determine optimal binding conditions. Calcium was one of the components which was varied to study binding between Nell2 and the α_{1B} subunit since the interaction with Syntaxin 1A was calcium sensitive (Sheng et al., 1994). Calcium gradients were established based on physiological calcium concentration and the in-vitro studies previously characterized with the calcium-dependent vesicle docking/fusion process (McEnery et al., 1998). Other variables included the detergents used in cell lysis (NP-40, TritonX-100, Tween-20) and binding/washing/elution conditions (salt concentration, buffers, incubation times). Some PKC binding proteins are also substrates of the kinase and some co-

immunoprecipitation experiments were carried out under phosphorylating conditions (Mochly-Rosen, 1995).

The yeast two-hybrid system is a sensitive and specialized method used to identify protein-protein interactions that occur within the yeast nucleus. The sensitivity of the yeast two-hybrid system allows detection of transient interactions and binding resulting from non-covalent interactions. These interactions are difficult to demonstrate by most standard protocols such as co-immunoprecipitation. The use of harsh detergents used to isolate expressed proteins in these assays destroys the interaction, although milder detergents such as NP-40 were used.

When expressing proteins in cells it is possible that the level of protein expression required to demonstrate a physical interaction has not been reached. It is also possible that a third interacting protein expressed in the yeast nucleus may be involved in the interaction which may not be expressed in the cell lines used. The interaction between Nell2 and Bait 4 may have required modifications to the proteins such as phosphorylation. Other protein-protein interactions with the α_{1B} subunit have proven to be calcium sensitive or voltage sensitive that may involve conformational differences in the channel. It is possible that the yeast nucleus supported a channel conformation, phosphorylation state or calcium environment which favored an interaction with Nell2. It appears that the interaction may be complex due to the fact that the standard techniques of identifying protein interactions have not been successful.

Future research to determine a direct physical interaction between the N-type calcium channel and Nell2 should be focused on sensitive techniques such as surface plasmon resonance (SPR) detection of biophysical interactions using the Biacore 3000 (http://www.biacore.com/indexanim.html). SPR technology can determine the kinetics of an

interaction, the concentration at which proteins interact, determine multi-protein complexes and it is possible to vary the binding conditions in real-time. Changes in calcium concentrations or changing phosphorylation conditions can be monitored as the interaction occurs. Using more sensitive techniques like SPR allow detection of non-covalent interactions and may help elucidate the necessary components for the Nell2/α_{1B} interaction.

Modulation of N-type Channels

Previous studies in HEK cells showed that application of PMA (100 to 400 nM) caused a 50 to 60 % up-regulation of N-type calcium channel currents (Zamponi et al., 1997, Hamid et al., 1999). In the present study, PKC peak up-regulation was induced by 25 nM PMA. The data also suggest that PMA has a time dependent bi-phasic effect in that it enhanced N-type current amplitudes at concentrations between 5 and 200 nM and inhibited currents at higher concentrations. N-type calcium channels are more sensitive to PMA when co-expressed with Nell2, shifting the dose response for PMA leftward. Only 2.5 nM PMA (a ten fold decrease in concentration) was required for the same increase in current as that observed with N-type channels alone at 25 nM PMA. Co-expression of antisense Nell2 in HEK cells expressing N-type channels abolished induced increase in N-type current. Taken together, the data suggests that in HEK cells, PKC modulation of N-type channels requires the presence of Nell2.

Antisense Nell2 experiments indicate Nell2 is required for upregulation of N-type calcium channels in HEK cells by the PKC pathway. Although antisense experiments are widely used in research today, the loss of protein expression needs to be confirmed. It is also possible that the introduction of the antisense construct affects other cell processes and loss of PKC upregulation and Nell2 expression must be correlated. In order to demonstrate loss of Nell2 expression in HEK cells, Western blots or cell staining should be carried out in the future.

It has been known for some time that N-type channels undergo inhibition when a G-protein pathway becomes activated (Dunlap and Fischbach, 1981; Swartz, 1993; Swartz et al., 1993, Hille, 1994; Stea et al., 1995; Bourinet et al., 1996). It has recently been determined that $G_{\beta/\gamma}$ subunits are responsible for the inhibition (Zhang et al., 1996, De Waard et al., 1997; Herlitze et al., 1997) and that activated $G_{\beta/\gamma}$ binds directly to the α_{1B} subunit (Patil et al., 1996; Zamponi and Snutch, 1998a). Dissociation of the $G_{\beta/\gamma}$ subunit occurs by application of a strong depolarizing prepulse and results in loss of inhibition of the calcium channel current called "facilitation" (Bean, 1989, Elmslie et al., 1992; Kasai, 1991, Patil et al., 1996). Co-expression of Nell2 with N-type channels in HEK cells resulted in an approximate 50 % decrease in average current density compared to wild type. Application of a strong depolarizing prepulse resulted in a robust increase in peak current activity in a manner reminiscent of that observed after activation of G-protein coupled receptors. Overall, these data are consistent with a Nell2-mediated tonic inhibition of N-type calcium channels by endogenous G-protein $\beta\gamma$ subunits.

To further investigate this possibility, co-expression of the carboxyl terminal fragment of the β adrenergic receptor kinase (βARKct) provided an effective $G_{\beta\gamma}$ sink by binding free $G_{\beta\gamma}$. Co-expression of βARKct resulted in a dramatic decrease in the Nell2-mediated G-protein inhibition. It has been shown that a C-terminal deletion (the last 381 amino acids) of the α_{1B} subunit can still undergo G-protein inhibition (Zamponi, 2001). This region contains the Nell2 binding site (Bait 4) and deletion of this region was predicted to disrupt Nell2-mediated G-protein inhibition of N-type channels. This was not the case and the C-terminal α_{1B} deletion underwent robust tonic G-protein inhibition in the absence of Nell2, suggesting that the carboxyl terminal region of the channel might be inhibitory to G-protein modulation. It was therefore

difficult to determine whether Nell2 binding to the channel and Nell2 modulation of the channel are distinct and separate events.

Over expression of a peptide corresponding to the Bait 4 region of the N-type channel carboxyl tail was carried out and the prepulse effect observed with Nell2 was dramatically attenuated. The Bait 4 peptide did not interfere with G-protein inhibition induced by over-expression of exogenous $G_{\beta 1\gamma 2}$, consistent with a mechanism in which the Bait peptide prevented formation of a Nell2-calcium channel complex rather than interfering with G-protein inhibition in general.

Deletion mutants of Nell2 were made to determine the region important for the Nell2-mediated G-protein enhanced inhibition of N-type channels. The first 20 amino acids were shown to abolish this effect. The construct was expressed in HEK cells and cell staining showed the same membrane localization as the full-length clone, despite having the potential signal sequence deleted. Thus, the N-terminal portion of Nell2 may comprise the minimal domain required for enhanced G-protein inhibition of the N-type channel.

Contributions of Nell2 and N-type Channels to Neurotransmission

It has been hypothesized that neurotransmitter release is a coordinated event involving between 50 to 100 different proteins (Augustine et al., 1999). Several distinct steps occur within this process and include translocation of vesicles from reserve pools to active zones, transition of docked vesicles to primed vesicles, fusion of vesicles to the membrane and finally, exocytosis. Calcium concentration has been shown to be a regulatory mechanism in the complex conformations of the presynaptic machinery involved in neurotransmission events (Hilfiker et al., 1999). Neurotransmitter release occurs within 200 μsec of an action potential and at least at some terminals, clustered N-type channels supply the 100 to 200 μM intracellular calcium

required to trigger exocytosis (Barrett and Stevens, 1972, Pumplin et al., 1981, Pumplin, 1983, Robitaille et al., 1990, Zucker, 1993).

A number of *in vitro* studies have led to a model suggesting that calcium concentration is key to the physiological events leading to neurotransmission (Catterall, 1999). Syntaxin is a key member in the traffic and fusion of vesicles and interacts with many proteins including nsec1 (Hata et al., 1993b, Garcia et al., 1994), CSP (Wu et al., 1999b), syntaphilin (Lao et al., 2000), SNAP-25 (Sollner et al., 1993, Chapman et al., 1995), sec6/8 (Hsu et al., 1996), tomosyn (Fujita et al., 1998), Munc-13 (Betz et al., 1997), synaptotagmin (Bennett et al., 1992; Chapman et al., 1995) and the N-type calcium channel (Bezprozvanny et al., 1995).

In vitro calcium concentrations of less than 10 μM, syntaxin, SNAP-25 and N-type channels form a complex which increases in affinity as calcium reaches 10 to 20 μM. Syntaxin-1A looses affinity for channel binding at higher calcium concentrations (>100 μM) and syntaxin favors binding to synaptotagmin, leading to synaptic vesicle release events (Catterall, 1999, Hilfiker et al., 1999). Shifting between resting and vesicle docking states are calcium-dependent and the identified calcium sensors include Doc2, synaptotagmin and Munc13 (Orita et al., 1995; Sudhof and Rizo, 1996, Naito et al., 1997).

Further regulation of the sequential interactions within presynaptic microdomains has been demonstrated with the SNARE proteins syntaxin, SNAP-25 and nSec-1 (Jarvis and Zamponi, 2001). PKC-dependent phosphorylation of the syntaxin Synprint site or co-expression with SNAP-25 or nSec-1 antagonizes syntaxin induced N-type channel inactivation, although the syntaxin-enhanced G-protein inhibition is unaffected. More recently, the cysteine string-proteins (CSPs) have been shown to modulate N-type channels by enhancing G-protein-dependent

inhibition similar to that for syntaxin (Magga et al., 2000). A model has been postulated where CSPs act as chaperones to enhance $G_{\beta\gamma}$/N-type channel complex formation and syntaxin or SNAP25/N-type channel formation (**Figure 45**).

PDZ domains function in forming large protein complexes, some of which have been identified to play important roles in neuronal functions such as presynaptic and postsynaptic organization.

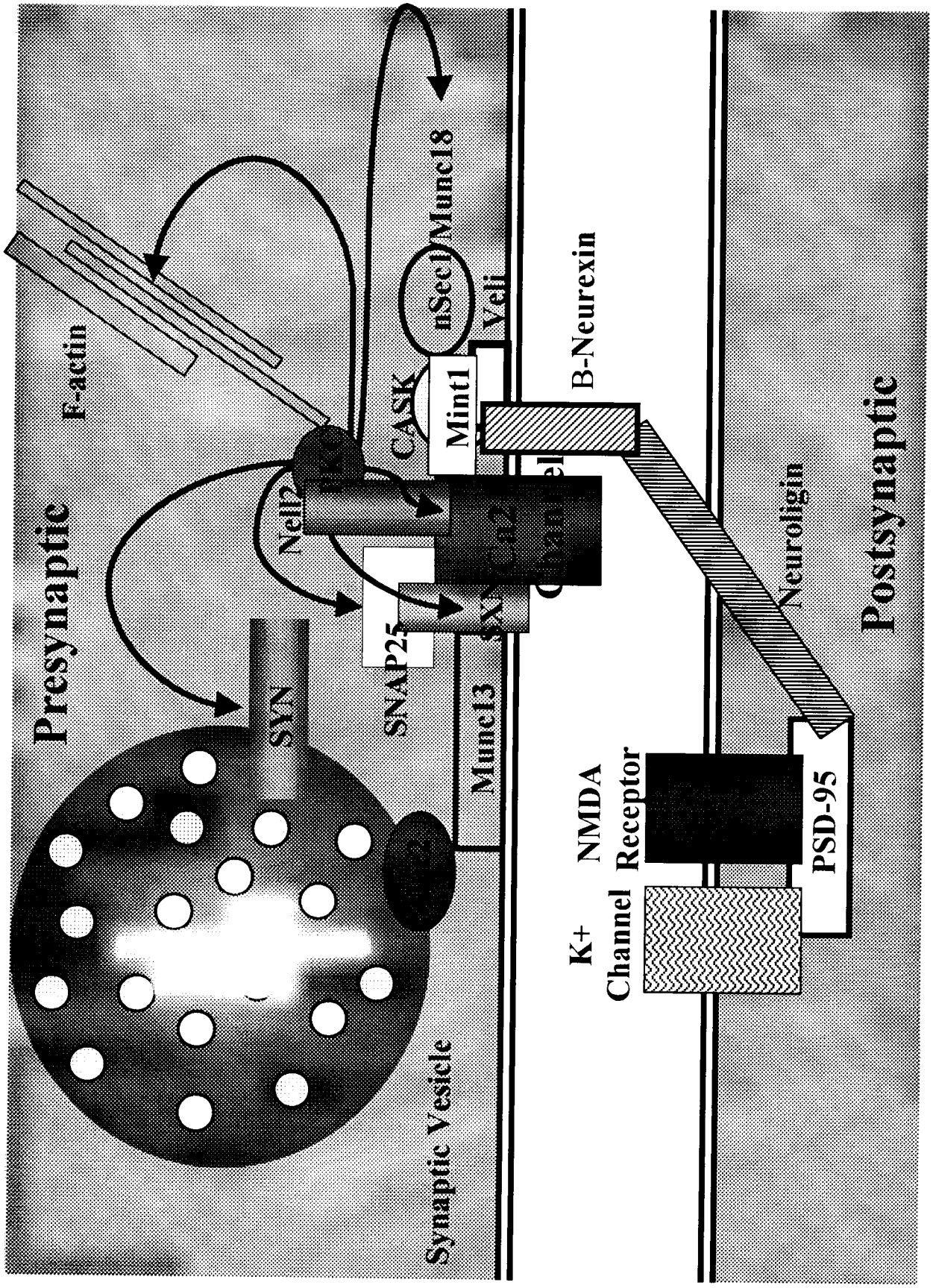
N-type calcium channels may be a member of such a complex since the α_{1B} subunit contains a novel type III PDZ binding domain at the C-terminus which binds a PDZ domain in Mint 1 (Maximov et al., 1999). Modulation by G-proteins is further organized by anchoring the $G_{\beta\gamma}$ subunit through syntaxin (Jarvis et al., 2000), and Nell2 likely anchors PKC to the N-type calcium channel. Other members of the complex could include clustering molecules and linker proteins to form complexes with receptors such as opioid receptors. To date, presynaptic N-type channels have been shown to be linked to postsynaptic NMDA receptors through a complex involving PSD-95/Neuroligin/β-Neurexin (Maximov et al., 1999).

Currently, the most defined role for N-type channels in the nervous system is their participation in neurotransmission. The rapid influx of calcium ions into the presynaptic neuron triggers neurotransmitter release. The G-protein pathway slows channel opening and results in decreased neurotransmitter release. Nell2 appears to enhance G-protein inhibition of N-type calcium channels and therefore may lead to a reduction in neurotransmitter release. Antisense experiments indicate that Nell2 is also necessary for PKC modulation. PKC modulation of N-type channels increases channel activity leading to increased intracellular calcium concentration and neurotransmitter release.

Figure 45. Nell2/N-type Channel Complex.

Combining the *in vitro* data of vesicle release machinery (reviewed by Catterall, 2000), modular adaptor proteins (Maximov et al., 1999) and previous studies along with the work of this thesis involving Nell2 (Kuroda et al., 1999; Kuroda and Tanizawa, 1999; Oyasu et al., 2000, Guthrie, 2001) a model has been proposed which links presynaptic and postsynaptic cells through neurotransmission processes based on calcium concentration and phosphorylation events. Although many protein-protein interactions are necessary for vesicle release, only a select few are shown in this model to emphasize the significance of the Nell2/N-type channel interaction. N-type calcium channels have been shown to associate with the SNARE proteins syntaxin-1A, SNAP-25 and synaptotagmin (Catterall, 2000). The interaction with syntaxin-1A and SNAP-25 is calcium dependent. This model is depicting a low calcium concentration situation, at higher calcium concentrations binding is less efficient and the interaction between N-type calcium channels and synaptotagmin is favored (Catterall, 1999; Catterall, 2000). Syntaxin interacts with Munc13 (Betz et al., 1997). Munc13 binds phorbol ester or diacylglycerol and thought to parallel the PKC pathway in neurotransmitter regulation (Betz et al., 1998). Munc13 binds Doc2, the interaction is enhanced upon binding phorbol ester or diacylglycerol (Kazanietz et al., 1995), and is affected by calcium concentration (Mochida et al., 1998). Synaptotagmin, Doc2 and Munc13 are all believed to be calcium sensors (Orita et al., 1995; Sudhof and Rizo, 1996, Naito et al., 1997). N-type channels have been shown to bind Mint1 and CASK (Maximov et al., 1999). Mint1 can bind CASK and Veli modular adaptor proteins, and nSec1/Munc18 (Maximov et al., 1999, Biederer and Sudhof, 2000). CASK can bind β-neurexin (Hata et al., 1996). β-neurexin associates with neuroligin which can bind PSD-95 (Irie et al., 1997). PSD-95 is a scaffold protein which binds NMDA receptors and potassium channels (Kim et al., 1995; Kornau et al., 1995). Nell2 binds the C-terminus of the α_{1B} subunit and PKC (Kuroda and Tanizawa, 1999, Guthrie,

2001). PKC can bind F-actin (Blobe et al., 1996). PKC substrates include F-actin, N-type calcium channels, nSec1/Munc18 and the SNARE proteins.


Presynaptic
F-actin
SYN
SNAP25
Munc13
Synaptic Vesicle
Nell2
CASK
Mint1
nSec1
Munc18
Veli
B-Neurexin
Neuroligin
K+
Channel
NMDA
Receptor
PSD-95
Postsynaptic

Anchoring PKC may not only allow phosphorylation of the N-type channel, but could enhance neurotransmission independent of calcium entry by acting to increase the number of release sites, releasable vesicles or altering calcium sensitivity of vesicles (Hilfiker and Augustine, 1999; Vaughan et al., 1998). Alternatively, Nell2 binding to PKC may render the enzyme inactive and thus favoring G-protein-dependent modulation of the channel and decreased ability to release neurotransmitter. Although results varied with Nell2 and PKC modulation in the work discussed here, since the G-protein and PKC pathway are very tightly coupled, it is clear that Nell2 plays a role in both processes and may therefore directly participate in N-type channel-mediated neurotransmission.

Voltage-independent modulation of N-type channels involve kinase-dependent phosphorylation (Dunlap, 1998). G-proteins are thought to carry out both voltage-dependent and voltage-independent forms of inactivation. One G-protein model suggests that G-proteins have the ability to shift the channel from a "willing" state to a "reluctant" state (Herlitze et al., 2001). During the willing state, N-type calcium channels activate more rapidly at negative membrane potentials, whereas in the reluctant state, activation is slow and requires more significant depolarization of membrane potential (Bean, 1989b). Prepulse facilitation is carried out by application of a strong depolarizing prepulse, which reverses G-protein modulation and thought to be the channel switching from a reluctant state to a willing state (Marchetti et al., 1986; Tsunoo et al., 1986, Bean, 1989; Elmslie et al., 1990).

The N-type channel appears to be in the reluctant state at rest whereas the P/Q-type channel is in the willing state (Herlitze et al., 2001). If the P/Q-type channel is expressed without a β subunit, the channel resembles N-type, tonically in the reluctant state at rest. Activation of the PKC pathway shifts the tonically inhibited N-type channel into the willing state. Therefore, N-type

channels are more sensitive to PKC modulation since at rest they are inhibited. This observation appears isoform specific since the rbB-I isoform is in the reluctant state at rest, but the rbB-II isoform is in the willing state similar to the P/Q-type channel (Herlitze et al., 2001). The amino acid residue responsible for isoform differences in resting state has been identified. A glutamate in the IS3 domain is thought to interact with a positive charge in the IS4 voltage sensor producing a reluctant state (Qin et al., 1997). Therefore, removing the C-terminus of the α_{1B} should not remove G-protein enhancement observed by the Nell2 interaction because the channel is tonically inhibited at rest.

Another interesting model postulated by Jarvis et al. (2000) suggests a feedback mechanism whereby the N-type channel is indirectly inhibited by the P/Q-type channel through a syntaxin-dependent pathway involving the direct interaction between syntaxin and $G_{\beta\gamma}$ subunits. It was previously demonstrated that the P/Q-type channel was able to upregulate the expression of syntaxin-1A (Sutton et al., 1999). Syntaxin can interact directly with both the P/Q- and N-type channels through the Synprint site (Sheng et al., 1994; Yokoyama et al., 1997) and has now been demonstrated to localize $G_{\beta\gamma}$ to the N-type channel and enhance G-protein inhibition (Jarvis et al., 2000). Similar to Nell2 which also enhances G-protein inhibition, syntaxin is not required for $G_{\beta\gamma}$ activity on N-type channels. Syntaxin itself directly modulates the N-type channel by shifting the inactivation potential in the negative direction (Bezprozvanny et al., 1995; Bezprozvanny et al., 2000; Jarvis et al., 2000) and slows inactivation (Degtiar et al., 2000).

The PKC pathway antagonizes the G-protein pathway at the I-II linker of the α_{1B} subunit in that phosphorylation of residue Thr^{422} blocks $G_{\beta\gamma}$ binding (Hamid et al., 1999). PKC phosphorylation of the Synprint site can also inhibit syntaxin binding (Yokoyama et al., 1997) and thus also

antagonize the G-protein pathway indirectly by inhibiting anchoring of the $G_{\beta\gamma}$ subunit to syntaxin (Jarvis et al., 2000). Although P/Q- and N-type channels are modulated by G-proteins and PKC, there is marked differences in the kinetic changes observed between the channel types (Bourinet et al., 1996; Stea et al., 1995; Zamponi et al., 1997). Most neurons express more than one type of calcium channel and the two channels mainly thought to be involved in neurotransmission may indirectly modulate each other through (Jarvis et al., 2000).
Calcium sensitive protein interactions not only include the SNARE proteins (Catterall, 1999) but may also include Nell2/N-type channels and Nell2/PKC by calcium binding EGF-like repeats (Kuroda and Tanizawa, 1999, Guthrie, 2001). Not all interactions are calcium dependent such as that of synaptotagmin and the α_{1B} subunit (Sheng et al., 1997) and other regulatory mechanisms such as phosphorylation can effect protein binding interactions in the synapse.

Phorbol esters can increase exocytosis by enhancing presynaptic calcium sensitivity and ultimately effecting priming or fusion events (Yawo, 1999). PKC and Munc13 are activated by diacylglycerol or phorbol esters and this process is also calcium dependent. Diacylglycerol is generated by hydrolysis of phosphoinositide by phopholipase C and requires a calcium concentration similar to that required for neurotransmitter release (Taylor et al., 1991; Zucker, 1996). The calcium required for diacylglycerol formation is believed to result from presynaptic calcium channels (Wakade et al., 1991).

PKC substrates are key to this process and some substrates include the SNARE proteins, nSec1/Munc18, F-actin and N-type calcium channels. Phosphorylation of SNAP-25 results in decreased binding to syntaxin-1A (Shimazaki et al., 1996) and disruption of the SNARE complex promoting vesicle release. PKC phosphorylation of nSec1/Munc18 also decreases binding to syntaxin-1A, increasing available unbound syntaxin-1A to form the SNARE complex

promoting vesicle release (Fujita et al., 1996). Synaptotagmin is also a PKC substrate as well as a calcium sensor and phosphorylation could effect calcium binding or formation of the SNARE complex (Hilfiker et al., 1999). Synaptotagmin is capable of binding syntaxin-1A and neurexin (Bennett et al., 1992; Hata et al., 1993a). Munc13 binds phorbol ester and interacts with both syntaxin-1A and Doc2 and enhances neurotransmitter release (Betz et al., 1998, Mochida, 1998). PKC not only phosphorylates, but also binds F-actin, which undergoes rearrangement of cytoskeletal matrix to allow vesicles to dock at vesicle release sites near the plasma membrane (Blobe et al., 1996).

When combining the complexity of the synaptic machinery proteins with that of the modular adaptor proteins and Nell2, a model was generated where tight regulation of the synapse couples N-type channels to postsynaptic NMDA/potassium channels to understand the importance of both calcium concentration and PKC phosphorylation events of neurotransmission (**Figure 45**). Although further research is required to elucidate all members of the complex and the regulation of protein-protein interactions within the complex, Nell2 could play an important role in localizing PKC to N-type calcium channels and presynaptic complexes.

Conclusions and Future Studies

Utilizing the yeast two-hybrid system, Nell2 has been shown to associate with the C-terminus of the α_{1B} subunit of the rat N-type calcium channel. Nell2 is an 816 aa PKC binding-protein and a family member of the **n**euronally expressed proteins containing **E**GF-**l**ike repeats (Nels, (Kuroda et al., 1999; Kuroda and Tanizawa, 1999). The Nell2/N-type channel interaction involves the last 362 aa of the α_{1B} subunit (residues 1972 to 2336 of rbB-I, Dubel et al., 1992) and a central 123 aa region of Nell2 (residues 477 to 600, Guthrie, 2001). The interaction has functional significance; Nell2 enhances both G-protein-dependent and PKC-dependent modulation of N-

type calcium channels expressed in HEK cells. The first 20 amino acids of Nell2 were found to be essential for the Nell2-mediated G-protein inhibition of N-type current. Antisense Nell2 experiments indicate that Nell2 is required for PKC modulation, enhancing the up-regulation of N-type channel current. Although a direct physical interaction has not been demonstrated outside of the yeast model system, mRNA and protein of both N-type channels and Nell2 appear to be localized together in various rat tissues and human and rat cell lines (Guthrie, 2001). This novel protein interaction will further the understanding of N-type channel physiology; in particular, provide a new mechanism for the regulation of N-type channel neurotransmission.

It has been demonstrated that the first 20 amino acids of Nell2 is important for the functional effect observed with enhanced G-protein modulation of N-type channels. This 20 amino acid region is highly hydrophobic and may contain a signal sequence, although cell staining of cells expressing the deletion mutant missing this region did not differ from the full-length clone. Other experiments indicated Nell2 is not a secreted protein in HEK cells. Further amino acid deletions would determine which residues are responsible for N-type modulation and may lead to a better understanding of Nell2 functional interaction with N-type calcium channels. The putative binding region of Nell2 (residues 477 to 600) to the α_{1B} subunit contains EGF-like repeats, domains known to be involved in protein-protein interactions. Dissection of the specific residues responsible for binding could isolate a unique domain essential for Nell2/α_{1B} subunit interaction.

Isoform specificity exists among the known calcium channel interacting-proteins. The P/Q-type calcium channel rbA α_{1A} subunit has been shown to bind synaptotagmin and SNAP25 and the BI α_{1A} subunit binds all three SNARE proteins as well as cysteine string-proteins (Csps), known to be associated with the synaptic vesicle (Rettig et al., 1996, Leveque et al., 1998). There are long

and short splice variants of α_{1A} and α_{1B} subunits, only the long form can interact with the PDZ domain of Mint1-1 (Maximov et al., 1999). Although the C-terminus of the α_1 subunits are diverse among channel types, there are two conserved regions among the α_{1A}, α_{1B} and α_{1E} subunits. The first is a proline rich region which contain six SH3-domain binding sites (PXXP, Feng et al., 1994). The SH3 domain in the human α_{1B} subunit consists of residues 2039 to 2194, which is within the Bait 4 sequence and also contains a $G_{\beta\gamma}$ binding site (Qin et al., 1997) and a secondary calcium channel β subunit binding site (Walker et al., 1998). The second conserved region amongst these channels is the DXXC-COOH, C-terminal end which has been hypothesized to be a novel PDZ domain binding consensus sequence (Kornau et al., 1997; Maximov et al., 1999).

Nell2 was shown to associate with the C-terminus of the α_{1B} subunit. Although this is a highly diverse region amongst calcium channels, it is possible that Nell2 interacts with other calcium channels. Since Nell2 affects G-protein and PKC-dependent modulation, it would be interesting to determine whether Nell2 can interact with other calcium channels that undergo these types of modulation. Functional studies involving co-expression of Nell2 with other calcium channels may reveal whether this protein can interact and modulate other calcium channels or if it is N-type channel specific.

The variety and distribution of ion channels in the neuronal plasma membrane is critical in order to carry out the important function of electrical signaling, but the mechanisms underlying the targeting of particular classes of channels to specific membrane sites are poorly understood. Identification of novel protein interactions and domains responsible for binding have been the focus of research into ion channel localization. Recent studies have identified a new class of

protein-protein interaction mediated by PDZ domains. Shaker-type potassium channels and NMDA receptors by are clustered by PSD-95 through PDZ domains. Notably, new PDZ domains have recently been identified and their roles have expanded from the initial scaffold protein hypothesis to protein modulator.

This has been shown with cystic fibrosis transmembrane conductance regulator (CFTR) whereby channel gating is modulated by two PDZ domain containing protiens, the Na+/H+ exchanger regulatory factor (NHERF) and the CFTR-associated protein 70 kDa (CAP70) (Bezprozvanny and Maximov, 2001). Protein-protein interactions with CFTR result in upregulation of channel activity when co-expressed with NHERF (Raghuram et al., 2001) or CAP70 (Wang et al., 2000). This is also the case for Homer, originally thought to cluster metabotropic glutamate receptors through Ena/VASP homology 1 domain (Brakeman et al., 1997), and now believed to regulate function of the receptor (Tu et al., 1998; Xiao et al., 1998).

Other clustering-proteins include rapsyn, which localizes nicotinic acetylcholine receptors through a zinc finger to the membrane (Bezakova and Bloch, 1998). Glycine and GABA receptors are clustered by gephyrin (Betz, 1998; Essrich et al., 1998) which is now thought to be mediated by a third protein, GABA receptor-associated protein (Chen et al., 2000; Kennedy, 2000). AMPA receptors are clustered through PDZ domains by GRIP/ABP (Srivastava et al., 1998; Wyszynski et al., 1998). It is not clear how calcium channels are localized or clustered to the membrane, although it has been suggested the auxiliary β subunit plays a role (Brice et al., 1997; Chien et al., 1995). It is possible that Nell2 anchors calcium channels to the membrane through f-actin since the PKCβII isoform binds and phosphorylates f-actin (Blobe et al., 1996). Perhaps a complex between N-type channel/Nell2/PKC/f-actin acts to stabilize channel conformation.

Nell2 as a Future Drug Target

Proteins which bind PKC are being considered as potential drug targets for specific neuronal pathologies and the use of PKC activators and inhibitors are already being used in the treatment of several diseases such as amyotrophic lateral sclerosis and ischemia (Manev et al., 1990). Additionally, deficient translocation of PKC into the brain of Alzheimer's patients has been shown to result in interference with amyloid precursor protein processing (Govoni et al., 1993; Nitsch and Growdon, 1994). It is considered possible that PKC binding-proteins contribute to the impairment in Alzheimer's disease and research has focused on factors that control PKC localization (Wang et al., 1994).

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Appendix

Figure 1. PCR Strategies.

All polymerase chain reactions (PCR) were carried out using a Perkin Elmer Cetus DNA Thermal Cycler (model #P4589). PCR reactions were initiated with a "hot start" whereby Taq polymerase (BRL) was added at 74°C to ensure that no polymerase activity occurred during reaction set up. Denaturation was at 95°C for 5 minutes at the beginning of the experiment and was also done in each cycle of the experiment. Seven cycles were carried out 5°C below the melting temperature (TM) complimentary region for annealing temperature of the oligonucleotide (the lowest temperature between the 2 oligonucleotides was used). The TM was determined by TM = 2 X (T+A) + 4 X (G+C) for oligonucleotides less than 30 bp, and TM = 81.5 + 0.41 (%GC) - 675/N (N was the length of the oligonucleotide in bp) for oligonucleotides greater than 30 bp in length. The TM complimentary was the region of the oligonucleotide that was complimentary or homologous to the template. The annealing and extension time ranged from 30 seconds to 3 minutes, dependent upon the size of the fragment being amplified. Twenty-five cycles were carried out at an annealing temperature 5°C below the TM of the entire oligonucleotide. At the end of the amplification step, an extension cycle at 72°C for 7 minutes was preformed prior to 4°C soaking.

A 50 µl reaction typically contained 5 µl 10 X PCR Buffer (200 mM Tris pH 8.4, 500 mM KCl) (BRL), 2 or 3 µl $MgCl_2$ (50 mM) (BRL), 5 µl 10X dNTP mix (2.5 mM of each dATP, dGTP, dCTP and dTTP) (BRL), 1 µl DNA template (1 ng/µl), 1 µl forward/sense oligonucleotide (20 pmol/µl), 1 µl reverse/antisense oligonucleotide (20 pmol/µl), 3 µl Dimethyl Sulfoxide (DMSO) (Fisher), 0.5 µl T4 Taq Polymerase (5 U/µl) (BRL), up to 50 µl with dH20. Taq Polymerase was added at 74°C during the thermocycle to eliminate inaccurate primer annealing. A $MgCl_2$

gradient using 2, 3, 4 or 5 µl/reaction was usually carried out. Reactions were topped with mineral oil to avoid loss of solution during heating.

Standard PCR

74°C	Add Taq Polymerase to reactions.	
95°C	5 min.	
94°C	30 sec. (denaturation)	
42°C	1.5 min. (annealing)	7 cycles
72°C	1.5 min. (extension)	
94°C	30 sec.	
70°C	1.5 min.	25 cycles
72°C	1.5 min.	
70°C	7 min.	
4°C	soak	

Touch-up PCR

PCR reactions were prepared as above except that the annealing temperature was altered at every second cycle by increasing temperature by 2 or 3 degrees until the final cycle which was typically at 5°C below the TM of the entire oligonucleotide (lower value of the 2 oligonucleotides used).

74°C	Taq polymerase was added to reactions.	
95°C	5 min.	
94°C	1.5 min.	
38°C	1.5 min.	2 cycles
72°C	1.5 min.	
94°C	1.5 min.	
40°C	1.5 min.	2 cycles
72°C	1.5 min.	
94°C	1.5 min.	
42°C	1.5 min.	2 cycles
72°C	1.5 min.	
94°C	1.5 min.	
44°C	1.5 min.	2 cycles
72°C	1.5 min.	

94°C	1.5 min.	
46°C	1.5 min.	2 cycles
72°C	1.5 min.	
94°C	1.5 min.	
48°C	1.5 min.	25 cycles
72°C	1.5 min.	
72°C	7 min.	
4°C	soak	

Touch-down PCR

PCR was carried out whereby the cycles were initiated at 12°C above the TM of the entire oligonucleotide and then repeated 2 times with a decreasing annealing temperature. The last cycle was repeated 25 times and was typically 5°C below the TM of the oligonucleotides used.

74°C	Taq polymerase was added to reactions.	
95°C	5 min.	
94°C	1.5 min.	
48°C	1.5 min.	2 cycles
72°C	1.5 min.	
94°C	1.5 min.	
46°C	1.5 min.	2 cycles
72°C	1.5 min.	
94°C	1.5 min.	
44°C	1.5 min.	2 cycles
72°C	1.5 min.	
94°C	1.5 min.	
42°C	1.5 min.	2 cycles
72°C	1.5 min.	

94°C	1.5 min.	
40°C	1.5 min.	2 cycles
72°C	1.5 min.	
94°C	1.5 min.	
38°C	1.5 min.	25 cycles
72°C	1.5 min.	
72°C	7 min.	
4°C	soak	

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The reverse oligonucleotide was used with RNA to carry out reverse transcription (RT) which generates a DNA template and can be used in PCR to amplify regions of a gene. Twenty pmol of reverse oligonucleotide was incubated with 1 μg of RNA at 90°C for 2 minutes. The sample was put on ice and microfuged briefly. Eight μl of reverse transcription master mix was added (5 mM $MgCl_2$, 1 X first strand buffer (BRL), 1 mM dNTP (BRL), 0.5 μl RNasin (BRL), Reverse Transcriptase (Superscript II, BRL) and dH_2O). Samples were incubated on ice for 10 minutes and then at 37°C for 1 hour. Heating samples to 99°C for 2 minutes ends the reaction. Samples were incubated on ice and PCR mix was added (4 mM $MgCl_2$, 1 X PCR buffer (BRL), 6% DMSO, 20 pmol forward olignucloetide, 0.25 μl Taq polymerase (BRL) and dH_2O). PCR was carried out using different protocols (standard, touch-up or touch-down) depending on the oligonucleotides used and the size of the fragment being amplified.

Figure 2. α_{1B} Subunit Oligonucleotide Map.

Seven regions of the α_{1B} subunit were generated as baits to look for protein-protein interactions in the yeast two-hybrid system (**Table 6, Figure 6**). The oligonucleotides used for PCR are shown (double underline) in relation to the four transmembrane domains (bolded underline) on the rbB-I α_{1B} subunit isoform (Dubel et al., 1992).

ATGGTCCGCTTCGGGGACGAGCTAGGCGGCCGCTATGGGGGCACCGGCGGCGGGGAGCGGGC

HG107

TCGGGGCGGCGGGGCCGGCGGGGCCGGTGGCCCGGGCCAGGGGGGTCTGCCGCCGGGCCAGC

GGGTCCTGTACAAGCAGTCCATTGCGCAACGCGCACGGACCATGGCCCTGTACAACCCCATCC

CAGTCAAGCAGAACTGCTTCACCGTCAACCGCTCGCTCTTCGTCTTCAGCGAGGACAACGTCG

TCCGCAAATATGCTAAGCGCATCACCGAATGGCCGCCCTTCGAATATATGATCCTGGCCACCA

HG108 I S1

TCATCGCCAACTGTATTGTCCTGGCCCTGGAGCAGCACCTCCCTGATGGGGACAAGACTCCCA

TGTCTGAACGACTGGATGACACGGAACCTTACTTCATCGGCATCTTTTGCTTCGAGGCGGGCA

I S2

TCAAGATCATAGCTCTGGGCTTCGTGTTCCACAAAGGCTCCTACCTCCGGAATGGCTGGAACG

TCATGGACTTCGTGGTGGTCCTCACAGAGATTCTTGCCACAGCTGGAACTGACTTTGATCTGC

I S3

GCACCCTGAGGGCTGTGCGTGTGCTTAGGCCCCTGAAGTTGGTGTCTGGAATTCCAAGCTTGC

I S4

AGGTGGTGCTCAAGTCCATCATGAAGGCCATGGTCCCGCTGCTGCAGATCGGGCTGCTGCTCT

TCTTCGCCATCCTCATGTTCGCTATCATCGGCCTCGAGTTCTATATGGGCAAATTCCATAAGGC

I S5

CTGCTTCCCCAACAGCACAGATGCAGAGCCTGTGGGTGACTTTCCTTGTGGCAAGGAGGCCCC

TGCTCGTCTGTGTGACAGTGACACCGAATGCCGGGAGTACTGGCCAGGACCCAACTTTGGCAT

CACCAATTTTGACAACATCCTGTTTGCCATCTTGACCGTGTTCCAGTGTATCACCATGGAGGGC

TGGACTGACATCCTCTACAATACAAATGATGCGGCCGGCAACACGTGGAACTGGTTGTACTTC

ATCCCCCTCATCATCATTGGCTCCTTCTTCATGCTCAACCTGGTGCTCGGTGTGCTTTCAGGAG

I S6

AGTTTGCCAAAGAGCGGGAGCGAGTCGAGAACCGCCGTGCCTTCCTGAAGCTCCGCAGGCAG

HG109

CAGCAGATTGAGCGAGAACTGAATGGGTACTTGGAGTGGATCTTCAAGGCGGAGGAAGTCAT

GTTGGCAGAGGAGGACAAGAACGCAGAAGAGAAGTCCCCTTTGGATGCAGTGTTGAAGAGAG

CTGCTACCAAGAAGAGCCGAAATGACCTCATCCATGCAGAAGAGGGGGAGGACCGGTTTGTA

GACCTCTGTGCTGCTGGGTCTCCCTTTGCTCGTGCCAGCCTCAAGAGTGGGAAGACAGAGAGC

TCATCGTACTTCCGGAGGAAGGAGAAGATGTTCCGGTTCCTTATCCGTCGTATGGTGAAAGCA

HG110

CAGAGCTTCTACTGGGTGGTACTGTGCGTGGTGGCCCTGAACACGTTGTGTGTGGCCATGGTA

II S1

CACTATAATCAGCCTCAGCGGCTTACCACTGCACTGTACTTTGCAGAGTTTGTTTTCCTGGGTC

TCTTCCTCACAGAGATGTCCCTGAAGATGTACGGTCTAGGGCCCAGAAGCTACTTCCGGTCTT

II S2

CCTTCAACTGCTTTGACTTTGGGGTGATTGTGGGGAGTATCTTTGAAGTAGTCTGGGCTGCCAT

II S3

CAAGCCAGGAACCTCCTTCGGAATCAGTGTGCTGCGGGCTCTCCGACTGCTGAGGATTTTCAA

II S4

AGTCACCAAGTATTGGAACTCCCTGAGGAACCTGGTTGTTTCCCTCCTCAACTCCATGAAGTC

CATCATCAGCCTTCTCTTCCTGCTTTTCCTTTTCATTGTGGTCTTCGCTCTGTTGGGGATGCAGC

II S5

TGTTTGGGGGACAGTTCAACTTTCAAGATGAGACTCCAACCACCAATTTTGATACCTTCCCAG

CTGCCATCCTCACTGTGTTTCAGATTCTGACAGGAGAGGACTGGAATGCAGTCATGTATCATG

GGATTGAGTCACAAGGAGGAGTCAGCAAAGGCATGTTTTCATCCTTTTACTTCATCGTCCTGA

CACTGTTTGGAAACTACACCCTGTTGAACGTTTTCTTGGCCATTGCTGTGGACAACCTTGCCAA

II S6 HG100

TGCCCAGGAGTTGACCAAGGATGAAGAGGAGATGGAAGAGGCAGCCAATCAGAAGCTTGCTC

TTCAGAAGGCCAAAGAAGTAGCTGAAGTCAGCCCCATGTCTGCTGCCAACATCTCCATTGCTG

CCAGGCAGCAGAACTCGGCCAAGGCGCGCTCAGTATGGGAGCAGCGGGCCAGTCAGCTAAGG

CTCCAGAACCTGCGTGCCAGCTGTGAGGCACTGTACAGTGAGATGGACCCGGAGGAGCGCCT

GCGTTATGCCAGCACGCGCCACGTGAGGCCAGACATGAAGACACACATGGACCGACCCCTAG

TGGTGGAACCTGGTCGGGATGGCCTGCGGGGACCCGCCGGGAACAAGTCAAAGCCTGAGGGC

ACGGAGGCCACCGAAGGTGCGGATCCACCACGCCGACACCACCGGCATCGTGATAGGGACAA

GACCTCAGCCTCAACCCCTGCTGGAGGCGAACAGGACAGGACAGACTGCCCAAAGGCCGAAA

HG101

GCACCGAGACCGGGGCCCGGGAGGAACGTGCGCGCCCTCGTCGAAGTCACAGCAAGGAGGCT

CCAGGGGCTGACACACAAGTGCGTTGTGAGCGCAGTAGACGTCACCACCGGCGCGGATCCCC

GGAGGAGGCCACTGAACGGGAACCTCGGCGCCACCGTGCCCACCGGCACGCACAGGACTCAA

GCAAGGAAGGCAAGGAGGGCACTGCACCGGTGCTTGTACCCAAGGGCGAGCGTCGCGCAAG

ACATCGAGGCCCGCGTACGGGCCCCCGTGAGACAGAGAACAGTGAGGAGCCCACACGCAGGC

ACCGTGCAAAGCATAAGGTGCCACCAACACTTGAGCCCCCAGAGAGGGAGGTTGCAGAGAAG

GAGAGCAACGTGGTGGAAGGGGATAAGGAAACTCGAAATCACCAGCCCAAGGAACCTCGCT

GTGACCTGGAGGCCATTGCGGTTACAGGCGTGGGCTCTCTGCACATGCTGCCCAGCACCTGTC

TCCAGAAAGTGGACGAACAGCCAGAGGATGCAGACAACCAGCGTAATGTCACCCGGATGGGC

AGTCAGCCCTCAGACCCCAGCACCACTGTGCATGTCCCAGTGACACTGACAGGCCCTCCCGGG

GAGGCCACTGTAGTTCCCAGTGCTAACACGGACCTGGAAGGCCAAGCGGAGGGCAAGAAGGA

GGCAGAGGCTGACGATGTGCTGAGAAGAGGCCCCAGGCCCATCGTTCCCTACAGTTCCATGTT

CTGCCTCAGCCCCACCAACCTACTCCGTCGCTTCTGCCATTACATTGTGACCATGCGGTACTTT

HG102

GAGATGGTGATTCTTGTGGTCATCGCCTTGAGCAGCATTGCCCTGGCTGCTGAGGATCCCGTG

III S1

CGGACCGACTCATTCCGGAACAATGCTCTGAAGTACATGGACTACATCTTTACAGGAGTCTTC

ACCTTTGAGATGGTCATAAAGATGATAGACTTGGGCCTGCTGCTGCACCCTGGGGCCTACTTC

III S2

CGGGACCTGTGGAACATTCTGGACTTCATTGTTGTCAGTGGAGCCCTGGTGGCATTTGCATTCT

III S3

CGAGCTTCATGGGAGGATCCAAAGGGAAAGACATCAATACCATCAAGTCTCTGAGAGTCCTG

CGAGTCCTGCGGCCCCTCAAGACCATCAAGCGGCTGCCTAAACTCAAGGCTGTGTTTGACTGT

III S4

GTGGTGAACTCTCTGAAGAATGTCTTGAACATCCTGATCGTCTACATGCTCTTCATGTTTATAT

III S5

TTGCCGTCATCGCCGTCCAACTCTTCAAAGGGAAGTTCTTTTACTGCACTGATGAGTCCAAGG

AGCTGGAGCGGGACTGCAGGGGTCAGTATTTGGATTATGAGAAGGAAGAGGTAGAAGCCCAG

CCAAGGCAGTGGAAGAAATATGACTTCCACTATGACAATGTGCTCTGGGCCTTGCTGACTCTG

TTTACGGTGTCCACAGGAGAGGGGTGGCCCATGGTGCTGAAACACTCTGTGGACGCCACCTAT

GAGGAGCAGGGGCCAAGCCCCGGGTTTCGGATGGAGCTTTCCATCTTCTATGTGGTCTACTTT

GTGGTCTTCCCTTTTTTCTTTGTCAACATCTTTGTGGCCTTGATCATCATCACCTTCCAGGAGCA

III S6

HG107

GGGGGACAAGGTGATGTCTGAGTGCAGTCTGGAAAAGAATGAGAGGGCTTGCATTGACTTTG

CCATCAGCGCCAAACCCCTGACACGGTACATGCCTCAGAACAAGCAGTCGTTCCAGTATAAG

ACATGGACATTTGTGGTCTCTCCACCCTTTGAGTACTTCATTATGGCCATGATAGCCCTCAACA

HG108

IV S1

CAGTGGTGCTGATGATGAAGTTCTACGATGCCCCTTATGAGTACGAGCTGATGCTGAAGTGCT

TGAACATCGTCTTCACATCCATGTTCTCTCTGGAGTGCATCCTGAAGATCATCGCCTTCGGGGT

IV S2

GTTGAACTACTTCAGAGATGCCTGGAACGTCTTTGACTTTGTCACTGTTTTGGGAAGTATTACT

IV S3

GATATTTTAGTAACGGAGATTGCGAACAACTTCATCAACTTGAGCTTCCTTCGCCTCTTCCGGG

CAGCACGGCTGATCAAGCTCTGTCGCCAGGGCTACACCATCCGCATCTTGTTATGGACCTTTG

IV S4

TCCAGTCCTTTAAGGCGCTGCCCTACGTGTGCCTCCTCATTGCCATGCTGTTCTTCATCTACGC

IV S5

CATCATCGGCATGCAGGTTTTTGGAAACATTGCCCTTGATGATGGCACCAGCATCAACCGACA

CAACAACTTCCGGACATTTCTGCAAGCCTTAATGCTGTTGTTCAGGAGTGCCACTGGGGAGGC

CTGGCACGAAATCATGCTGTCTTGCCTGGGCAACCGGGCCTGCGACCCACATGCCAACGCCAG

CGAATGCGGGAGCGACTTTGCCTATTTTTATTTTGTCTCCTTCATCTTCCTCTGTTCCTTTCTGA

IV S6

TGCTGAACCTCTTTGTTGCTGTGATCATGGACAATTTCGAATACCTCACGCGGGATTCTTCCAT

CCTAGGGCCGCACCACCTCGATGAATTCATTCGCGTCTGGGCTGAATACGACCCAGCTGCGTG

HG103

TGGGCGCATCAGTTACAATGACATGTTTGAGATGCTGAAACACATGTCCCCACCTCTGGGTTT

GGGGAAGAAATGCCCGGCTCGAGTTGCATACAAGCGCCTGGTTCGAATGAACATGCCCATAT

CCAATGAGGACATGACGGTACACTTTACATCCACACTGATGGCCCTCATCCGGACGGCACTGG

AGATCAAGCTTGCCCCAGCGGGGACAAAACAGCACCAATGTGATGCTGAGCTGAGGAAGGAG

ATCTCTTCTGTGTGGGCTAATCTGCCCCAGAAGACTCTGGACTTACTGGTGCCACCCCACAAA

CCTGACGAGATGACAGTGGGGAAGGTCTATGCGGCTCTCATGATATTTGACTTCTACAAACAG

AACAAAACCACCAGAGATCAGACTCACCAAGCTCCTGGAGGCCTGTCCCAGATGGGTCCTGTT

HG104

TCCCTATTCCATCCTCTGAAGGCCACCCTGGAGCAGACACAGCCCGCTGTGCTCCGAGGAGCT

CGGGTTTTCCTTCGACAAAAGAGTGCAACTTCCCTCAGCAATGGGGGCGCCATACAAACCCAG

GAAAGTGGCATCAAGGAGTCCCTGTCCTGGGGCACGCAGAGGACCCAGGACGTACTTTATGA

GGCCAGAGCACCTCTAGAACGTGGCCATTCTGCAGAGATCCCTGTGGGGCAGCCAGGAGCAC

TGGCTGTAGATGTCCAGATGCAGAACATGACATTGAGAGGACCGGATGGGGAGCCCCAGCCT

HG105

GGCCTGGAGAGCCAAGGCCGAGCGGCCTCTATGCCACGCCTGGCGGCAGAAACACAGCCGGC

CCCTAATGCCAGCCCCATGAAGCGCTCCATCTCCACACTGGCTCCACGCCCGCATGGGACTCA

GCTTTGCAACACAGTCCTGGACCGGCCACCTCCTAGCCAGGTGTCCCATCACCACCACCACCG

CTGCCACCGGCGCAGGGACAAGAAGCAGAGGTCCCTGGAAAAGGGGCCCAGCCTGTCTGTTG

ACACAGAAGGTGCACCAAGTACTGCTGCAGGATCTGGCCTGCCCCATGGAGAAGGGTCCACA

GGCTGCCGGCGGGAGCGTAAGCAAGAGCGAGGCCGGTCCCAGGAGCGGAGGCAGCCCTCCTC

CTCTTCTTCAGAGAAGCAGCGCTTCTATTCCTGTGACCGCTTTGGGAGCCGGGAGCCCCCACA

ACCTAAGCCCTCCCTCAGTAGCCACCCCATATCGCCAACAGCGGCACTAGAGCCAGGACCCCA

CCCGCAGGGCAGTGGTTCCGTTAATGGGAGCCCCTTGATGTCAACATCTGGTGCTAGCACGCC

GGGCCGAGGTGGGCGGAGGCAGCTCCCCCAGACTCCCCTGACCCCACGCCCCAGCATCACCT

ACAAGACGGCCAATTCCTCGCCTGTCCACTTTGCTGAGGGTCAGAGTGGCCTTCCAGCCTTCT

CCCCTGGCCGTCTCAGCCGCGGCCTTTCTGAACACAATGCCCTGCTCCAGAAAGAGCCCCTGA

GCCAGCCTCTAGCTTCTGGCTCCCGCATTGGCTCTGACCCTTACCTAGGGCAGCGTCTGGACA

GTGAGGCCTCTGCCCACAACCTGCCTGAGGATACACTCACCTTTGAAGAGGCCGTGGCCACCA

ACTCTGGCCGCTCCTCCAGGACTTCCTATGTGTCCTCCCTCACTTCCCAATCCCACCCTCTCCG

CCGTGTACCCAATGGCTACCACTGCACTTTGGGACTCAGCACCGGCGTCCGGGCGCGGCACAG

CTACCACCACCCAGACCAGGATCACTGGTGCTAG

HG106

Figure 3. Oligonucleotide Summary.

1. TM is the melting temperature of an oligonucleotide determined via the sequence. For small oligonucleotides (less than 30 base pairs), TM=2x(T+A) + 4x(G+C) and for large oligonucleotides (30 to 60 base pairs), TM=81.5+0.41(%GC)-675/N, where N is the length of the oligonucleotide in base pairs. 2. TMc is the complimentary TM (TMc), region of the oligonucleotide which is a compliment of the DNA sequence it primes to. 3. Forward (sense) vs reverse (antisense) oligonucleotide. 4. Vector oligonucleotide positions are given in nucleotide base pairs (bp). 5. The (-) symbol indicates these oligonucleotides were not used in PCR and TM was not required. 6. HG90 oligonucleotide is downstream the stop codon and the location is indicated in nucleic acid number (base pair, bp). 7. Rat B4.67 clone is a yeast two-hybrid (YIIH) DNA fragment which encodes the PKC-BP Nell2 and also some of the 3' non-coding region of the gene which was found to interact with bait 4 (C-terminus of the α_{1B} subunit). 8. Rat Nell2 clone was the product of RT-PCR generating a full length clone that differs by 7 amino acids and an elongated C-terminus from the PKC-BP Nell2 (U48245). The Rat Nell2 clone sequence generated has not been published, no accession number available. 9. SP6 is a universal reverse oligonucleotide used for priming sequence across many vector multiple cloning sites in the reverse orientation. Location is not shown. 10. T7 forward oligonucleotide is used for priming sequence across many vector multiple cloning sites in the forward/sense orientation. Location is not shown.

NAME	AMINO ACID POSITION/CLONE (ACCESSION NUMBER)	TM[1]	TMc[2]	SEQUENCE (5' to 3')	F or R[3]
HG1	1-9/Rat β_3 Calcium Channel Subunit (P54287).	94	76	TGC GGC CGC GCC ACC ATG GAC TAC AAG GAC GAC GAT GAC AAG TAT GAC GAC TCC TAC GTG	F
HG2	289-296/Rat β_3 Calcium Channel Subunit (P54287).	60	-	CTC TTT GAC GAA GAC GAT GAT	R
HG5	1-6/Rat Mu Opioid Receptor (L20684).	81	60	ACC ATT GAA TTC ATG GAC AGC AGC ACC GGC	F
HG6	55-60/Rat Mu Opioid Receptor (L20684).	82	60	AAT AAT GTC GAC CTA TTA GGT CTG AGG GCA CAG GCT	R
HG8	1-6/Mouse 5-HT1c (CAA51031).	80	56	AGG ATG GAA TTC ATG GTG AAC CTG GGC ACT	F
HG9	55-60/ Mouse 5-HT1c (CAA51031).	79	52	AAT AAT GTC GAC CTA TTA TGA AAG TGC TGG CCA GTT	R
TPS202	1-6/Rat α_{1A} Calcium Channel Subunit (P54282)	90	66	CCA TCA GAT CTA TGG CCC GCT TTG GAG AC	F
TPS203	43-48/Rat α_{1A} Calcium Channel Subunit (P54282)	85	66	CCA TCA GAT CTT CCT CTG CGC TCC GGG CTG	R
TPS204	1-6/Rat α_{1E} Calcium Channel Subunit (Q07652).	92	68	CCA TCA GAT CTA TGG CTC GCT TCG GGG AG	F
TPS205	53-58/Rat α_{1E} Calcium Channel Subunit (Q07652).	90	58	CCA TCA GAT CTC CGG ACT GGT ATG GGG TT	R
TPS206	1-6/Rat α_{1C} Calcium Channel Subunit (AAA42016).	82	50	CCA TCA GAT CTA TGG TCA ATG AAA ACA CG	F
TPS207	46-51/Rat α_{1C} Calcium Channel Subunit (AAA42016).	94	62	CCA TCA GAT CTG AAC AGT GCT GCC CCT GG	R
TPS208	1-6/Rat α_{1D} Calcium Channel Subunit (P27732).	74	50	CCA TCA GAT CTA TGA AAA AAA TGC AGC ATC	F

TPS209	47-52/Rat α_{1D} Calcium Channel Subunit (P27732).	86	54	CCA TCA GAT CTA GAC AGG ACG GTT TGC TT	R
TPS210	1-6/Rat α_{1B} Calcium Channel Subunit (M92905).	92	60	CCA TCA GAT CTA TGG TCC GCT TCG GGG AC	F
TPS211	38-44/Rat α_{1B} Calcium Channel Subunit (M92905).	89	70	CCA TCA GAT CTG GAC CCG CTG GCC CGG CGG	R
HG100	711-715/Rat α_{1B} Calcium Channel Subunit (M92905).	76	64	CCA TCG AAT TCG ACA ACC TTG CCA AT	F
HG101	874-878/Rat α_{1B} Calcium Channel Subunit (M92905).	82	46	GGT AGG TCG ACC TTT GGG CAG TCT GT	R
HG102	1144-1148/Rat α_{1B} Calcium Channel Subunit (M92905).	82	46	GGT AGG TCG ACC CGC ATG GTC ACA AT	R
HG103	1728-1732/Rat α_{1B} Calcium Channel Subunit (M92905).	62	44	CCA TCG AAT TCA TTC GCG TC	F
HG104	1871-1875/Rat α_{1B} Calcium Channel Subunit (M92905).	84	44	GGT AGG TCG ACG TGA GTC TGA TCT CT	R
HG105	1972-1976/Rat α_{1B} Calcium Channel Subunit (M92905).	78	46	CCA TCG AAT TCG CTG TAG ATG TCC AG	F
HG106	2333-2336/Rat α_{1B} Calcium Channel Subunit (M92905).	82	38	CCA TCG TCG ACC TAG CAC CAG TGA TC	R
HG107	1419-1424/Rat α_{1B} Calcium Channel Subunit (M92905).	78	56	CCA TCG AAT TCA TCA CCT TCC AGG AG	F
HG108	1469-1473/Rat α_{1B} Calcium Channel Subunit (M92905).	84	52	CCA TCG TCG ACT GGA GAG ACC ACA AA	R
HG107A	1-5/Rat α_{1B} Calcium Channel Subunit (M92905).	82	50	CCA TCG AAT TCA TGG TCC TCC GCT TCG GG	F
HG108A	91-95/Rat α_{1B} Calcium Channel Subunit (M92905).	84	48	CCA TCG TCG ACC GGC CAT TCG GTG AT	R
HG109	357-361/Rat α_{1B} Calcium Channel Subunit (M92905).	76	44	CCA TCG AAT TCG GAG AGT TTG CCA AA	F

HG110	479-483/Rat α_{1B} Calcium Channel Subunit (M92905).	80	44	CCA TCG TCG ACC TGT GCT TTC ACC AT	R
HG200A	1-5/Rat Syntaxin A (AF217191).	85	46	CCA TCG CTA GCG AAT TCG CCA CCA TGA AGG ACC GAA TT	F
HG201	285-289/Rat Syntaxin A (AF217191).	76	44	CCA TCG AAT TCC TAT CCA AAG ATG CC	R
HG202	83-89/Rat Syntaxin A (AF217191).	50	-	GAA GAC AGC GAA CAA AG	F
HG203	231-237/Rat Syntaxin A (AF217191).	50	-	ATG ATC TCA CTG TGC CT	R
HG87	1-6/Rat PKC-BP Nell2 (U48245).	86	46	ATG CTG CTA GCG AAT TCG CCA CCA TGG AAT CCC GGG TA	F
HG90	2559-2580 bp[6]/Rat PKC-BP Nell2. (U48245).	78	50	AGC TGC GGC CGC TTT AAA TGA GAA ATC ATT CTT	R
HG92	75-80/Rat PKC-BP Nell2 (U48245).	54	-	GAG CGG TTT TTC CAG AAG	F
HG93	155-160/Rat PKC-BP Nell2 (U48245).	56	-	TTA GCC TTC AGT GCC TCC	F
HG94	235-140/Rat PKC-BP Nell2 (U48245).	54	-	CAT GGG CTT GTG CAG AAA	F
HG95	315-320/Rat PKC-BP Nell2 (U48245).	58	-	GCT AAA TCG GCT CCA GCG	F
HG96	395-400/Rat PKC-BP Nell2 (U48245).	58	-	TGC AAA GGT TAT GAC TTC	F
HG97	475-480/Rat PKC-BP Nell2 (U48245).	52	-	ATC GAC GAT TAC TCG TGT	F
HG98	555-560/Rat PKC-BP Nell2 (U48245).	52	-	GAC ATT GAT GAG TGC TCT	F
HG99	635-640/Rat PKC-BP Nell2 (U48245).	52	-	AAC TGC ACA GGG GAC TGC	F
HG120	1972-1976/Rat α_{1B} Calcium Channel Subunit (M92905).	92	46	TTA ACG TCG ACG CCA CCA TGG ACT ACA AGG ACG ACG ATG ACA AGG CTG TAG ATG TCC AG	F
HG121	2333-2336/Rat α_{1B} Calcium Channel Subunit (M92905).	78	50	CCT TGG AAT TCC TAG CAC CAG TGA TC	R
HG122	328-333/Rat PKC-BP Nell2 (U48245).	92	48	CCT CGG CGG CCG CGC CAC CAT GAA GGG AGC CAC C	F
HG123	808-813/Rat PKC-BP Nell2 (U48245).	90	50	GGA TCG AAT TCG GCT GGA GGC ACT GTG G	R

HG85	49-53/Rat PKC-BP Nell2 (U48245).	70	46	ATC AAG CTT CCC ATT ATG CAG TCC	R
HG86	714-719/Rat PKC-BP Nell2 (U48245).	60	-	TGG GCC CAG GAT TGC CGT	F
HG84	814-819/Rat PKC-BP Nell2 (U48245).	52	-	GAG CTG TGA AGT TAA CTG	F
HG35	1-6/Rat PKC-BP Nell2 (U48245).	90	52	TTG AAT TCG CCA CCA TGG ACT ACA AGG ACG ACG ATG ACA AGA TGG AAT CCC GGG TAT TA	F
HG36	94-99/Rat PKC-BP Nell2 (U48245).	56	-	AAG GTC ACC AGA ATT GTG AA	R
HG28	2618-2636 bp/Rat B4.67 YIIH Clone[7].	60	-	GCG GCC AGC CAA TGC AAC	F
HG29	2915-2932 bp/Rat B4.67 YIIH Clone.	58	-	CCT CGC TGG TTC AGG AAC	R
HG30	2869-2886 bp/Rat B4.67 YIIH Clone.	54	-	AGC TCA GAC TGT TCC ACA	F
HG79	2661-2706 bp/Rat B4.67 YIIH Clone.	87	-	GTT TCC TCA GTG ATG TTC CTC AAA GCT CTA CAG AGC CGT AAT CGC	R
JM91	176-182/Rat Alpha-tubulin (VO1227).	70	-	GAG GTG TCC ACG GCT GTG GTG	F
JM92	242-248/Rat Alpha-tubulin (VO1227).	64	-	AGG GCT CCA TCG AAA CGC AG	R
JM93	219-224/Rat Alpha-tubulin (VO1227).	56	-	ATT GAG CGC CCA ACC TAC	F
HG33	314-319/Rat Nell2 Clone[8].	80	56	AAT ATT GAA TTC ATG CCG GCT AAA TCG GCT CC	F
HG34	385-390/Rat Nell2 Clone.	80	36	TTT AAT GTC GAC CTA GCT GTG AGA CAA GGC GA	R
HG40	249-253/Rat Nell2 Clone.	85	48	GGG AAT TCG CCA CCA TGT CGA AGA CGT CAG CC	F
HG41	253-259/Rat Nell2 Clone.	54	-	AGC TCT AGA CAA CTT GGC	R
HG42	474-478/Rat Nell2 Clone.	58	42	CCT CTA GAA TCG ACG ATT AC	F
HG43	450-454/Rat Nell2 Clone.	46	-	TGT TCT CAC GGC AGT	R
HG44	650-655/Rat Nell2 Clone.	72	56	TTG GCG CCA GAT CTG GGT GCT G	F

HG45	656-660/Rat Nell2 Clone.	92	48	AAG CGG CCG CTC AGC ACC TGT CGT TCT C	R
HG105F	1972-1976/Rat α_{1B} Calcium Channel Subunit (M92905).	200	46	CCA TCG AAT TCG CCA CCA TGG ACT ACA AGG ACG ACG ATG ACA AGG CTG TAG ATG TCC AG	F
HG106F	2332-2337/Rat α_{1B} Calcium Channel Subunit (M92905).	78	46	CCA TCT CTA GAC TAG CAC CAG TGA TC	R
HG17	707-711/Rat Nell2 Clone.	46	-	ACC GTG TAC AAC AGC	F
HG18	824-829/Rat Nell2 Clone.	172	50	ATT GCG GCC GCT TTC ACT TGT CAT CGT CGT CCT TGT AGT CCA GCT CCT GGA GGC A	R
HG76	21-25/Rat Nell2 Clone.	172	48	TAA GAA TTC GCC ACC ATG GAC TAC AAG GAC GAC GAT GAC AAG GGA CTT GGT GTG GAC	F
HG77	93-98/Rat Nell2 Clone.	44	-	TAA GTG GAT CTG TTT CAG	R
HG73	187-192/Rat Nell2 Clone.	72	52	ATT GGT GAC CAC ATT TTG GTT GGG A	F
HG71	101-107/Rat Nell2 Clone.	178	54	TAA GAA TTC GCC ACC ATG GAC TAC AAG GAC GAC GAT GAC AAG GTC ATT CTC TCC ATC CAC	F
HG72	362-366/Rat Nell2 Clone.	54	-	CAT GGT CTG ATC CTT GCA	R
HG74	175-180/Rat Nell2 Clone.	66	54	TAT CTA GAA GGC ATT TCC ACC AC 3'	R
HG75	255-260/Rat Nell2 Clone.	50	-	5' TTG TCT AGA GCT GAA CAA	F
HG115	1-5/B4.46 YIIH Clone.	172	48	TAA GAA TTC GCC ACC ATG GAC TAC AAG GAC GAC GAT GAC AAG ATC CTC CAG GAG GAC	F
HG116	220-226/B4.46 YIIH Clone.	100	45	AAA GCG GCC GCT CAG CAG AAC TTG TTG CAG TG	R
1C	889-894/Rat α_{1B} Calcium Channel Subunit (M92905).	56	-	CAA GAC CTC AGC CTC AAC	F

R27	925-931/Rat α_{1B} Calcium Channel Subunit (M92905).	58	-	AGC CTC CTT GCT GTG ACT T	R
1G	1002-1007/Rat α_{1B} Calcium Channel Subunit (M92905).	54	-	GTG AGA CAG AGA ACA GTG	F
R25A	1081-1086/Rat α_{1B} Calcium Channel Subunit (M92905).	58	-	TCT GCA TCC TCT GGC TGT T	R

pMT2 Reverse	1131-1151 bp[4]/pMT2 vector (Invitrogen).	66	-	CAG CCT TCA CGC TAG GAT TGC	R
pAdVA Forward	752-770 bp/pAdVA vector (GenVec).	58	-	CTC TTC CGT GGT CTG GTG	F
PGex4T-1 Forward	855-876 bp/pGex4T-1 vector (pharmacia).	60	-	GCA TGG CCT TTG CAG GGC	F
PGex4T-1 Reverse	976-997 bp/pGex4T-1 vector (pharmacia).	50	-	ATG TGT CAG AGG TTT TCA	R
pGAD10 Forward	2499-2516 bp/pGAD10 vector (Clonetech).	48	-	TAC CAC TAC AAT GGA TG	F
pGAD10 Reverse	2715-2732 bp/pGAD10 vector (Clonetech).	46	-	GAA AGA AAT TGA GAT GG	R
pAS2-1 Forward	5895-5911 bp/pAS2-1 Vector (Clonetech)	50	-	TCA TCG GAA GAG AGT AG	F
PET28a Forward	347-365 bp/PET28a(+) Vector (Novagen).	56	-	AGA TCT CGA TCC CGC GAA	F
PET28a Reverse	67-84 bp/PET28a(+) Vector (Novagen).	52	-	TGC TAG TTA TTG CTC AGC	R
SP6	Reverse Oligonucleotide[9].	72	-	CCT ATT CTA TAG TGT CAC CTA AAT GC	R
T7	Forward Oligonucleotide[10].	66	-	GTA AAT ACG ACT CAC TAT AGG GC	F
pMal-2 Forward	2586-2604 bp/pMAL-2 Vector (New England Biolabs Inc.)	56	-	GGT CGT CAG ACT GTC GAT	F
pTracer Forward	769-790 bp/pTracer-CMV2 Vector (Invitrogen).	70	-	CGC AAA TGG GCG GTA GGC GTG	F

Figure 4. Bait Sequences

Seven regions of the α_{1B} subunit were generated from the rbB-I isoform (accession M92905, Dubel et al., 1992) as Baits to look for protein-protein interactions in the yeast two-hybrid system (**Table 6, Figure 6**). A Bait of syntaxin-1A was also constructed (accession AF217191, Bennett et al., 1992). The oligonucleotides used for PCR are listed. The nucleotide sequence and protein sequence are shown.

BAIT 1

Bait 1, 504 base pairs and contains the first part of the II-III linker of the α_{1B} subunit.

Oligos HG100 forward/sense 5' CCA TCG AAT TCG ACA ACC TTG CCA AT 3'

HG101 reverse/antisens 5' GGT AGG TCG ACC TTT GGG CAG TCT GT 3'

Bait 1 DNA Sequence:

GAC AAC CTT GCC AAT GCC CAG GAG TTG ACC AAG GAT GAA GAG GAG ATG
GAA GAG GCA GCC AAT CAG AAG CTT GCT CTT CAG AAG GCC AAA GAA GTA
GCT GAA GTC AGC CCC ATG TCT GCT GCC AAC ATC TCC ATT GCT GCC AGG CAG
CAG AAC TCG GCC AAG GCG CGC TCA GTA TGG GAG CAG CGG GCC AGT CAG
CTA AGG CTC CAG AAC CTG CGT GCC AGC TGT GAG GCA CTG TAC AGT GAG ATG
GAC CCG GAG GAG CGC CTG CGT TAT GCC AGC ACG CGC CAC GTG AGG CCA
GAC ATG AAG ACA CAC ATG GAC CGA CCC CTA GTG GTG GAA CCT GGT CGG
GAT GGC CTG CGG GGA CCC GCC GGG AAC AAG TCA AAG CCT GAG GGC ACG
GAG GCC ACC GAA GGT GCG GAT CCA CCA CGC CGA CAC CAC CGG CAT CGT
GAT AGG GAC AAG ACC TCA GCC TCA ACC CCT GCT GGA GGC GAA CAG GAC
AGG ACA GAC TGC CCA AAG

Bait 1 Protein Sequence:

D N L A N A Q E L T K D E E E M E E A A N Q K L A L Q K A K E V A E V S P M S A A N
I S I A A R Q Q N S A K A R S V W E Q R A S Q L R L Q N L R A S C E A L Y S E M D P E
E R L R Y A S T R H V R P D M K T H M D R P L V V E P G R D G L R G P A G N K S K P
E G T E A T E G A D P P R R H H R H R D R D K T S A S T P A G G E Q D R T D C P K

BAIT 2

Bait 2 consists of 1314 base pairs, the entire II-III linker of the α_{1B} subunit.

Oligos HG100 5' CCA TCG AAT TCG ACA ACC TTG CCA AT 3'

HG102 5' GGT AGG TCG ACC CGC ATG GTC ACA AT 3'

Bait 2 DNA Sequence:

GAC AAC CTT GCC AAT GCC CAG GAG TTG ACC AAG GAT GAA GAG GAG ATG

GAA GAG GCA GCC AAT CAG AAG CTT GCT CTT CAG AAG GCC AAA GAA GTA

GCT GAA GTC AGC CCC ATG TCT GCT GCC AAC ATC TCC ATT GCT GCC AGG CAG

CAG AAC TCG GCC AAG GCG CGC TCA GTA TGG GAG CAG CGG GCC AGT CAG

CTA AGG CTC CAG AAC CTG CGT GCC AGC TGT GAG GCA CTG TAC AGT GAG ATG

GAC CCG GAG GAG CGC CTG CGT TAT GCC AGC ACG CGC CAC GTG AGG CCA

GAC ATG AAG ACA CAC ATG GAC CGA CCC CTA GTG GTG GAA CCT GGT CGG

GAT GGC CTG CGG GGA CCC GCC GGG AAC AAG TCA AAG CCT GAG GGC ACG

GAG GCC ACC GAA GGT GCG GAT CCA CCA CGC CGA CAC CAC CGG CAT CGT

GAT AGG GAC AAG ACC TCA GCC TCA ACC CCT GCT GGA GGC GAA CAG GAC

AGG ACA GAC TGC CCA AAG GCC GAA AGC ACC GAG ACC GGG GCC CGG GAG

GAA CGT GCG CGC CCT CGT CGA AGT CAC AGC AAG GAG GCT CCA GGG GCT

GAC ACA CAA GTG CGT TGT GAG CGC AGT AGA CGT CAC CAC CGG CGC GGA

TCC CCG GAG GAG GCC ACT GAA CGG GAA CCT CGG CGC CAC CGT GCC CAC

CGG CAC GCA CAG GAC TCA AGC AAG GAA GGC AAG GAG GGC ACT GCA CCG

GTG CTT GTA CCC AAG GGC GAG CGT CGC GCA AGA CAT CGA GGC CCG CGT

ACG GGC CCC CGT GAG ACA GAG AAC AGT GAG GAG CCC ACA CGC AGG CAC

CGT GCA AAG CAT AAG GTG CCA CCA ACA CTT GAG CCC CCA GAG AGG GAG

GTT GCA GAG AAG GAG AGC AAC GTG GTG GAA GGG GAT AAG GAA ACT CGA

AAT CAC CAG CCC AAG GAA CTT CGC TGT GAC CTG GAG GCC ATT GCG GTT ACA

GGC GTG GGC TCT CTG CAC ATG CTG CCC AGC ACC TGT CTC CAG AAA GTG GAC GAA CAG CCAGAG GAT GCA GAC AAC CAG CGT AAT GTC ACC CGG ATG GCC AGT CAG CCC TCA GAC CCC AGC ACC ACT GTG CAT GTC CCA GTG ACA CTG ACA GGC CCT CCC GGG GAG GCC ACT GTA GTT CCC AGT GCT AAC ACG GAC CTG GAA GGC CAA GCG GAG GGC AAG AAG GAG GCA GAG GCT GAC GAT GTG CTG AGA AGA GGC CCC AGG CCC ATC GTT CCC TAC AGT TCC ATG TTC TGC CTC AGC CCC ACC AAC CTA CTC CGT CGC TTC TGC CAT TAC ATT GTG ACC ATG CGG

Bait 2 Protein Sequence:

D N L A N A Q E L T K D E E E M E E A A N Q K L A L Q K A K E V A E V S P M S A A N I S I A A R Q Q N S A K A R S V W E Q R A S Q L R L Q N L R A S C E A L Y S E M D P E E R L R Y A S T R H V R P D M K T H M D R P L V V E P G R D G L R G P A G N K S K P E G T E A T E G A D P P R R H H R H R D R D K T S A S T P A G G E Q D R T D C P K A E S T E T G A R E E R A R P R R S H S K E A P G A D T Q V R C E R S R R H H R R G S P E E A T E R E P R R H R A H R H A Q D S S K E G K E G T A P V L V P K G E R R A R H R G P R T G P R E T E N S E E P T R R H R A K H K V P P T L E P P E R E V A E K E S N V V E G D K E T R N H Q P K E L R C D L E A I A V T G V G S L H M L P S T C L Q K V D E Q P E D A D N Q R N V T R M A S Q P S D P S T T V H V P V T L T G P P G E A T V V P S A N T D L E G Q A E G K K E A E A D D V L R R G P R P I V P Y S S M F C L S P T N L L R R F C H Y I V T M R

BAIT 3

Bait 3 contains 417 base pairs, the first part of the carboxy terminus of the α_{1B} subunit.

Oligos HG103 forward/sense 5' CCA TCG AAT TCA TTC GCG TC 3'

HG104 reverse/antisense 5' GGT AGG TCG ACG TGA GTC TGA TCT CT 3'

Bait 3 DNA Sequence:

GAA TTC ATT CGC GTC TGG GCT GAA TAC GAC CCA GCT GCG TGT GGG CGC ATC AGT TAC AAT GAC ATG TTT GAG ATG CTG AAA CAC ATG TCC CCA CCT CTG GGT TTG GGG AAG AAA TGC CCG GCT CGA GTT GCA TAC AAG CGC CTG GTT CGA ATG AAC ATG CCC ATA TCC AAT GAG GAC ATG ACG GTA CAC TTT ACA TCC ACA CTG ATG GCC CTC ATC CGG ACG GCA CTG GAG ATC AAG CTT GCC CCA GCG GGG ACA AAA CAG CAC CAA TGT GAT GCT GAG CTG AGG AAG GAG ATC TCT TCT GTG TGG GCT AAT CTG CCC CAG AAG ACT CTG GAC TTA CTG GTG CCA CCC CAC AAA CCT GAC GAG ATG ACA GTG GGG AAG GTC TAT GCG GCT CTC ATG ATA TTT GAC TTC TAC AAA CAG AAC AAA ACC ACC AGA GAT CAG ACT CAC

Bait 3 Protein Sequence:

E F I R V W A E Y D P A A C G R I S Y N D M F E M L K H M S P P L G L G K K C P A R V A Y K R L V R M N M P I S N E D M T V H F T S T L M A L I R T A L E I K L A P A G T K Q H Q C D A E L R K E I S S V W A N L P Q K T L D L L V P P H K P D E M T V G K V Y A A L M I F D F Y K Q N K T T R D Q T H

BAIT 4

Bait 4 consists of 1086 amino acids and contains the last part of the carboxy terminus of the α_{1B} subunit.

Oligos HG105 forward/sense 5' CC ATC GAA TTC GCT GTA GAT GTC CAG 3'

HG106 reverse/antisense 5' CC ATC GTC GAC CTA GCA CCA GTG ATC 3'

Bait 4 DNA Sequence:

GCT GTA GAT GTC CAG ATG CAG AAC ATG ACA TTG AGA GGA CCG GAT GGG GAG CCC CAG CCT GGC CTG GAG AGC CAA GGC CGA GCG GCC TCT ATG CCA CGC CTG GCG GCA GAA ACA CAG CCG GCC CCT AAT GCC AGC CCC ATG AAG CGC TCC ATC TCC ACA CTG GCT CCA CGC CCG CAT GGG ACT CAG CTT TGC AAC ACA GTC CTG GAC CGG CCA CCT CCT AGC CAG GTG TCC CAT CAC CAC CAC CAC CGC TGC CAC CGG CGC AGG GAC AAG AAG CAG AGG TCC CTG GAA AAG GGG CCC AGC CTG TCT GTT GAC ACA GAA GGT GCA CCA AGT ACT GCT GCA GGA TCT GGC CTG CCC CAT GGA GAA GGG TCC ACA GGC TGC CGG CGG GAG CGT AAG CAA GAG CGA GGC CGG TCC CAG GAG CGG AGG CAG CCC TCC TCC TCT TCT TCA GAG AAG CAG CGC TTC TAT TCC TGT GAC CGC TTT GGG AGC CGG GAG CCC CCA CAA CCT AAG CCC TCC CTC AGT AGC CAC CCC ATA TCG CCA ACA GCG GCA CTA GAG CCA GGA CCC CAC CCG CAG GGC AGT GGT TCC GTT AAT GGG AGC CCC TTG ATG TCA ACA TCT GGT GCT AGC ACG CCG GGC CGA GGT GGG CGG AGG CAG CTC CCC CAG ACT CCC CTG ACC CCA CGC CCC AGC ATC ACC TAC AAG ACG GCC AAT TCC TCG CCT GTC CAC TTT GCT GAG GGT CAG AGT GGC CTT CCA GCC TTC TCC CCT GGC CGT CTC AGC CGC GGC CTT TCT GAA CAC AAT GCC CTG CTC CAG AAA GAG CCC CTG AGC CAG CCT CTA GCT TCT GGC TCC CGC ATT GGC TCT GAC CCT TAC CTA GGG CAG CGT CTG GAC AGT GAG GCC TCT GCC CAC AAC CTG CCT GAG GAT ACA CTC ACC TTT GAA GAG GCC GTG GCC ACC AAC TCT GGC CGC TCC TCC AGG ACT TCC TAT GTG TCC TCC CTC ACT TCC CAA TCC CAC CCT CTC CGC CGT GTA CCC AAT GGC TAC CAC TGC ACT TTG GGA CTC AGC ACC GGC GTC CGG GCG CGG CAC AGC TAC CAC CAC CCA GAC CAG GAT CAC TGG TGC TAG

Bait 4 Protein Sequence:

A V D V Q M Q N M T L R G P D G E P Q P G L E S Q G R A A S M P R L A A E T

Q P A P N A S P M K R S I S T L A P R P H G T Q L C N T V L D R P P P S Q V S H H H H

H R C H R R R D K K Q R S L E K G P S L S V D T E G A P S T A A G S G L P H G E G S T

G C R R E R K Q E R G R S Q E R R Q P S S S S S E K Q R F Y S C D R F G S R E P P Q P K

P S L S S H P I S P T A A L E P G P H P Q G S G S V N G S P L M S T S G A S T P G R G G

R R Q L P Q T P L T P R P S I T Y K T A N S S P V H F A E G Q S G L P A F S P G R L S R

G L S E H N A L L Q K E P L S Q P L A S G S R I G S D P Y L G Q R L D S E A S A H N L P

E D T L T F E E A V A T N S G R S S R T S Y V S S L T S Q S H P L R R V P N G Y H C T L

G L S T G V R A R H S Y H H P D Q D H W C Stop

BAIT 5

Bait 5 contains the entire III-IV linker, 177 base pairs of the α_{1B} subunit.

Oligos HG107 forward/sense 5' CC ATC GAA TTC ATC ACC TTC CAG GAG 3'

HG108 reverse/antisense 5' CC ATC GTC GAC TGG AGA GAC CAC AAA 3'

Bait 5 DNA Sequence:

ATC ACC TTC CAG GAG CAG GGG GAC AAG GTG ATG TCT GAG TGC AGT CTG

GAA AAG AAT GAG AGG GCT TGC ATT GAC TTT GCC ATC AGC GCC AAA CCC CTG

ACA CGG TAC ATG CCT CAG AAC AAG CAG TCG TTC CAG TAT AAG ACA TGG

ACA TTT GTG GTC TCT CCA

Bait 5 Protein Sequence:

I T F Q E Q G D K V M S E C S L E K N E R A C I D F A I S A K P L T R Y M P Q N K Q S

F Q Y K T W T F V V S P

BAIT 6

Bait 6 contains 285 base pairs from the N-terminus of the α_{1B} subunit.

Oligos HG107A forward/sense 5' CC ATC GAA TTC ATG GTC CTC CGC TTC GGG 3'

HG108A reverse/antisense 5' CC ATC GTC GAC CGG CCA TTC GGT GAT 3'

Bait 6 DNA Sequence:

ATG GTC CGC TTC GGG GAC GAG CTA GGC GGC CGC TAT GGG GGC ACC GGC GGC GGG GAG CGG GCT CGG GGC GGC GGG GCC GGC GGG GCC GGT GGC CCG GGC CAG GGG GGT CTG CCG CCG GGC CAG CGG GTC CTG TAC AAG CAG TCC ATT GCG CAA CGC GCA CGG ACC ATG GCC CTG TAC AAC CCC ATC CCA GTC AAG CAG AAC TGC TTC ACC GTC AAC CGC TCG CTC TTC GTC TTC AGC GAG GAC AAC GTC GTC CGC AAA TAT GCT AAG CGC ATC ACC GAA TGG CCG

Bait 6 Protein Sequence:

M V R F G D E L G G R Y G G T G G G E R A R G G G A G G A G G P G Q G G L P P G Q R V L Y K Q S I A Q R A R T M A L Y N P I P V K Q N C F T V N R S L F V F S E D N V V R K Y A K R I T E W P

BAIT 7

Bait 7 contains 381 base pairs, the entire I-II linker of the α_{1B} subunit.

Oligos HG109 forward/sense 5' CC ATC GAA TTC GGA GAG TTT GCC AAA 3'

HG110 reverse/antisense 5' CC ATC GTC GAC CTG TGC TTT CAC CAT 3'

Bait 7 DNA Sequence:

GGA GAG TTT GCC AAA GAG CGG GAG CGA GTC GAG AAC CGC CGT GCC TTC CTG AAG CTC CGC AGG CAG CAG CAG ATT GAG CGA GAA CTG AAT GGG TAC TTG GAG TGG ATC TTC AAG GCG GAG GAA GTC ATG TTG GCA GAG GAG GAC AAG AAC GCA GAA GAG AAG TCC CCT TTG GAT GCA GTG TTG AAG AGA GCT GCT ACC AAG AAG AGC CGA AAT GAC CTC ATC CAT GCA GAA GAG GGG GAG GAC CGG TTT GTA GAC CTC TGT GCT GCT GGG TCT CCC TTT GCT CGT GCC AGC CTC AAG AGT GGG AAG ACA GAG AGC TCA TCG TAC TTC CGG AGG AAG GAG AAG ATG TTC CGG TTC CTT ATC CGT CGT ATG GTG AAA GCA CAG

Bait 7 Protein Sequence:

G E F A K E R E R V E N R R A F L K L R R Q Q Q I E R E L N G Y L E W I F K A E E V M L A E E D K N A E E K S P L D A V L K R A A T K K S R N D L I H A E E G E D R F V D L C A A G S P F A R A S L K S G K T E S S S Y F R R K E K M F R F L I R R M V K A Q

Syntaxin-1A

The Syntaxin Bait contains the entire coding region, 288 amino acids.

Oligos HG200A forward/sense CCA TCG CTA GCG AAT TCG CCA CCA TGA AGG ACC GAA TT 3'

HG201 reverse/antisense 5' CCA TCG AAT TCC TAT CCA AAG ATG CC 3'

Syntaxin DNA Sequence

ATG AAG GAC CGA ACC CAG GAG CTC CGC ACG GCC AAG GAC AGC GAT GAC GAC GAT GAT GTC ACT GTC ACT GTG GAC CGA GAC CGC TTC ATG GAT GAG TTC TTT GAA CAG GTG GAA GAG ATC CGG GGC TTT ATT GAC AAG ATT GCT GAG AAC GTG GAG GAG GTG AAG AGG AAA CACA GCG CCA TCC TGG CCT CCC CGA ACC

CCG ATG AGA AGA CCA AGG AGG AAC TGG AGG AGC TCA TGT CGG ACA TTA AGA AGA CAG CGA ACA AAG TTC GCT CCA AGC TAA AGA GCA TCG AGC AGA GCA TCG AGC AGG AGG AAG GTC TGA ACC GCT CGT CGG CGG ACC TGA GGA TCC GGA AGA CGC AGC ATT CCA CGC TGT CCC GAA AGT TTG TGG AGG TCA TGT CCG AGT ACA ACG CCA CTC AGT CAG ACT ACC GAG AAC GCT GCA AAG GGC GCA TCC AGA GGC AGC TGG AGA TCA CTG GCC GGA CCA CGA CCA GTG AGG AGT TGG AAG ACA TGC TGG AGA GTG GGA ATC CCG CCA TCT TTG CCT CTG GGA TCA TCA TGG ACT CCA GCA TCT CGA AGC AGG CCC TCA GTG AGA TCG AGA CCA GGC ACA GTG AGA TCA TCA AGT TGG AGA ACA GAC TCC GGG AGC TAC ACG ATA TGT TCA TGG ACA TGG CCA TGC TGG TGG AGA GCC AGG GGG AGA TGA TTG ACA GGA TCG AGT ACA ATG TGG AAC ACG CTG TGG ACT ACG TGG AGA GGG CCG TGT CTG ACA CCA AGA AGG CCG TCA AGT ACC AGA GCA AGG CAC GCA GGA AGA AGA TCA TGA TCA TCA TTT GCT GTG TGA TTC TGG GCA TCA TCA TCG CCT GCA CCA TCG GGG GCA TCT TTG GAT AGA TCC

Syntaxin Protein Sequence:

MKDRTQELRTAKDSDDDDDVTVTVDRDRFMDEFFEQVEEIRG FIDKIAENVEEVKRKHSAILASPNPDEKTKEELEELMSDIKKTA NKVRSKLKSIEQSIEQEEGLNRSSADLRIRKTQHSTLSRKFVEV MSEYNATQSDYRERCKGRIQRQLEITGRTTTSEELEDMLESGN PAIFASGIIMDSSISKQALSEIETRHSEIIKLENRLRELHDMFMD MAMLVESQGEMIDRIEYNVEHAVDYVERAVSDTKKAVKYQSK ARRKKIMIIICCVILGIIIACTIGGIFG

Figure 5. Rat Nell2 Oligonucleotide Map.

Oligonucleotides used in this project for rat Nell2 are shown (underlined).

1 ATGGAATCCC GGGTATTACT GAGAACGTTC TGCGTGATCC TCGGGCTCGA AGCGGTTTGG

HG35/HG87

61 GGACTTGGTG TGGACCCCTC CCTACAGATT GACGTCTTAT CAGAGTTAGA ACTTGGGGAG

HG76

121 TCCACAGCTG GAGTGCGCCA AGTCTCAGGA CTGCATAATG GGACGAAAGC CTTCCTCTTC

HG85

181 CAAGATTCCC CCAGAAGCAT AAAAGCACCC ATTGCTACAG CTGAGCGGTT TTTCCAGAAG

HG92

241 CTGAGGAATA AACACGAGTT CACAATTCTG GTGACCCTGA AACAGATCCA CTTAAATTCG

HG77

301 GAAGTCATTC TCTCCATCCA CCACTTGGAT CACAGGTACC TGGAACTGGA AAGCAGCGGC

HG71

361 CACCGGAATG AGATCAGACT GCATTACCGC TCTGGAACTC ACCGCCCGCA CACGGAAGTG

421 TTTCCTTATA TTTTGGCTGA TGCCAAGTGG CACAAGCTCT CCTTAGCCTT CAGTGCCTCC

HG93

481 CACTTAATTT TACACATCGA CTGCAACAAG ATCTATGAAC GAGTGGTGGA AATGCCTTCT

HG74

541 ACAGACTTGC CTCTGGGCAC CACATTTTGG TTGGGACAGA GAAATAACGC ACACGGGTAT

HG73

601 TTTAAGGGAA TAATGCAAGA TGTGCAATTA CTTGTCATGC CCCAGGGGTT CATCGCTCAG

661 TGCCCGGATC TTAATCGAAC CTGTCCAACA TGCAACGACT TCCATGGGCT TGTGCAGAAA

HG94

721 ATCATGGAGC TGCAGGACAT TTTATCGAAG ACGTCAGCCA AGTTGTCTAG AGCTGAACAA

HG40/HG41/HG75

781 CGAATGAACA GGCTGGATCA GTGCTACTGT GAGCGGACGT GCACCATGAA GGGAGCCACC

HG122

841 TACCGGGAGT TCGAGTCCTG GACAGACGGC TGCAAGAACT GCACATGCTT GAATGGGACC

901 ATCCAGTGCG AGACTCTGGT CTGCCCTGCT CCCGACTGCC CGGCTAAATC GGCTCCAGCG

HG33/HG95

961 TACGTGGATG GCAAGTGCTG TAAGGAGTGC AAGTCCACCT GCCAGTTCCA GGGGCGGAGC

1021 TACTTTGAGG GAGAAAGGAG CACAGTCTTC TCAGCTTCCG GAATGTGCGT CTTGTATGAA

1081 TGCAAGGATC AGACCATGAA GCTTGTTGAG AACGCCGGCT GCCCGGCTTT AGATTGCCCC

HG72

1141 GAGTCTCATC AGATCGCCTT GTCTCACAGC TGCTGCAAGG TTTGCAAAGG TTATGACTTC

HG34 HG96

1201 TGTTCTGAGA AGCATACATG CACGGAGAAC TCAGTCTGCA GGAACCTGAA CGACAGGGCA

1261 GTGTGCAGCT GCCGGGATGG TTTCCGGGCC CTCCGGGAGG ACAATGCCTA CTGTGAAGAC

1321 ATTGACGAGT GTGCAGAGGG GCGCCATTAC TGCCGTGAGA ACACCATGTG TGTGAACACA

HG43

1381 CCGGGCTCTT TCCTGTGTAT CTGCCAAACA GGGTACATCA GAATCGACGA TTACTCGTGT

HG42/HG97

1441 ACGGAACATG ACGAGTGCCT CACAAACCAG CACAACTGTG ACGAGAACGC TTTGTGCTTT

1501 AACACCGTTG GAGGTCACAA CTGCGTCTGC AAGCCTGGCT ACACTGGGAA TGGAACCACG

1561 TGCAAAGCTT TCTGCAAAGA CGGCTGCAGA AACGAAGGTG CCTGCATTGC TGCCAATGTC

1621 TGTGCTTGCC CACAAGGCTT CACCGGACCC AGCTGTGAGA CAGACATTGA TGAGTGCTCT

HG98

1681 GAGGGCTTTG TTCAGTGTGA CAGCCGTGCC AACTGCATTA ACCTGCCTGG GTGGTACCAC

1741 TGTGAGTGCA GAGATGGCTA CCATGACAAT GGGATGTTTG CGCCAGGTGG AGAATCCTGT

1801 GAAGATATTG ATGAATGTGG GACTGGGAGG CACAGCTGTG CCAATGACAC CATTTGCTTC

1861 AACTTGGACG GTGGCTACGA TTGCCGGTGT CCCCATGGAA AGAACTGCAC AGGGGACTGC

HG99

1921 GTGCACGACG GGAAAGTCAA ACACAACGGC CAGATCTGGG TGCTGGAGAA CGACAGGTGC

HG44/HG45

1981 TCTGTGTGTT CCTGCCAGAC TGGATTTGTT ATGTGTCGAC GGATGGTCTG TGACTGCGAA

2041 AACCCCACAG TTGACCTCTC CTGCTGCCCT GAGTGCGACC CAAGGCTGAG CAGCCAGTGC

2101 CTGCATCAAA ACGGGGAAAC CGTGTACAAC AGCGGTGACA CCTGGGTCCA GGATTGCCGT

HG17 HG86

2161 CAGTGCCGCT GCTTGCAAGG AGAAGTTGAC TGCTGGCCCC TGGCTTGCCC AGAGGTAGAG

2221 TGTGAATTTA GTGTCCTTCC TGAGAACGAG TGCTGCCCAC GCTGTGTCAC CGATCCTTGT

2281 CAGGCTGACA CCATCCGCAA TGACATCACC AAAACCTGCC TGGACGAGAT GAACGTGGTT

2341 CGCTTCACTG GGTCTTCCTG GATCAAGCAC GGCACGGAGT GCACCCTCTG CCAGTGCAAG

2401 AACGGCCACG TGTGCTGCTC AGTGGACCCA CAGTGCCTCC AGGAGCTGTG A

HG18/HG123/HG84/HG90

3' NON-TRANSLATED

2452 AGTTAACTGC CTCATGGGAG ATACCTGTTC AAAGAATGAT TTCTCATTTA AAAAGACCAA

2512 AAAACAAAAA AGAAAAAAAG TGATGTGCGG CCAGCCAATG CAACTGTGTC AATGGCTGGG

HG28

2572 CAGACTGATG GCGATTACGG CTCTGTAGAG CTTTGAGGAA CATCACTGAG GAAACCAGAT

2632 GGCAGTTCCG CCTTTACTGT TCCTGGGATC ACCTTATGGA GGAATGGCTG TGAATCACAG

2692 GCCTTGACAT CCCCAGCCCT GGAGAAGAAG CCTGAGCCCA TCAGCTCTGG GGAAGTCTCT

2752 CCCTCTCTCC CTCCCTCCAC AGGCACAGGA CATGTCCTAG CTCAGACTGT TCCACAGGCA

HG30

2812 CAGGACATGT CCTAGCTCAG ACTGTTCCTG AACCAGCGAG GTTCCTCACT GAAGCCATGG

HG29

2872 AATGAAAGGC AGTGAGTGAG CTATATTTTC AGAATCCAAG AAGCTGACAC ATCTGTACAG

2932 TGCACTCCGA ACCCTGAAAC AAGCTATTGT AATGATAAAA TACTGCACAG GCATGGTTAT

2992 GTAACATTTT CTAACCGG

Figure 6. Positive Yeast Two-hybrid Clone DNA Sequences.

DNA and translated protein sequences of yeast two-hybrid positive clones identified using the C-terminus of the α_{1B} subunit (bait 4), B4.6, B4.7, B4.15, B4.25, B4.66, B4.67, B4.69 and B4.84. Clones were sequenced in the pGAD10 library vector using pGAD10 forward and reverse oligonucleotides by manual sequencing. cDNA was translated to protein by Expasy's Translate Tool (http://www.expasy.org/tools/dna.html).

>pGAD10/B4.6

GAGTGCAAGTCCACCTGCCAGTTCCAGGGGCGGAGCTACTTTGAGGGAGAAAGCAG

CACAGTCTTCTCAGCTTCCGGAATGTGCGTCTTGTATGAATGCAAGGATCAGACCAT

GAAGCTTGTTGAGAACGCCGGCTGCCCGGCTTTAGATTGCCCCGAGTCTCATCAGAT

CGCCTTGTCTCACAGCTGCTGCAAGGTTTGCAAAGGTTATGACTTCTGTTCTGAGAA

GCATACATGCATGGAGAACTCAGTCTGCAGGAACCTGAACGACAGGGCAGTGTGCA

GCTGCCGGGATGGTTTCCGGGCCCTCCGGGAGGACAATGCCTACTGTGAAGACATTG

ACGAGTGTGCAGAGGGGCGCCATTACTGCCGTGAGAACACCATGTGTGTGAACACA

CCGGGCTCTTTCCTGTGTATCTGCCAAACAGGGTACATCAGAATCGACGATTACTCG

TGTACGGAACATGACGAGTGCCTCACAAACCAGCACAACTGTGACGAGAACGCTTT

GTGCTTTAACACCGTTGGAGGTCACAACTGCGTCTGCAAGCCTGGCTACACTGGGAA

TGGAACCACGTGCAAAGCTTTCTGCAAAGACGGCTGCAGAAACGGAGGTGCCTGCA

TTGCTGCCAATGTCTGTGCTTGCCCACAAGGCTTCACCGGACCCAGCTGTGAGACAG

ACATTGATGAGTGCTCTGAGGGCTTTGTTCAGTGTGACAGCCGTGCCAACTGCATTA

ACCTGCCTGGGTGGTACCACTGTGAGTGCAGAGATGGCTACCATGACAATGGGATG

TTTTGCGCCAGGTGGAGAATCCTGTGAAGATATTGATGAATGTGGGACTGGGAGGC

ACAGCTGTGCCAATGACACCATTTGCTTCAACTTGGACGGTGGCTACGATTGCCGGT

GTCCCCATGGAAAGAACTGCACAGGGACTGCGTGCACGACGGGAAAGTCAAACACC

ACGGCC

Translated Protein

ECKSTCQFQGRSYFEGESSTVFSASGMCVLYECKDQTMKLVENAGCPALDCPESHQIAL

SHSCCKVCKGYDFCSEKHTCMENSVCRNLNDRAVCSCRDGFRALREDNAYCEDIDECA

EGRHYCRENTMCVNTPGSFLCICQTGYIRIDDYSCTEHDECLTNQHNCDENALCFNTVG

GHNCVCKPGYTGNGTTCKAFCKDGCRNGGACIAANVCACPQGFTGPSCETDIDECSEG

FVQCDSRANCINLPGWYHCECRDGYHDNGMFCARWRIL

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CTGGCCTCACTGGCTGCACCACCCGCTGCTTCGAAGGCTGTGAGTGCGATGATCACT

TCCTGCTCTCCCAGGGTGTGTGCATTCCTGCCCAGGACTGCGGCTGTGTCCACAATG

GCCAATATGTGCCGGTGAACTCCTCCCTGATGTCCTCAGACTGCAGTGAACTCTGTT

ACTGCTCTGAAACCACCGGTCTGACATGCCGTGCAGCTGGCTGCCCATCTGGCCAGG

TCTGTGAGATCCAAGCTGGAGTCCGGCAGTGCCGGGCTGCTCATGGTCTCTGCTCCC

TCTCTGTGGGTGCCAACCTCACTACCTTCGATGGGGCTCTCAATACCATCAGCTCAC

CCGGTGTCTATGAGCTTTCTTCTCGCTGTCCGGGATTCCAGAAGAATGTGCCCTGGT

ACCGATTGCTTGCTGATGTCCAGCCTTGCCTTAACAATGACAAGATCGTGAGCAAGG

TCCACATCTTCTTCCAGGATGGCATGGTGACTGTGATCCCAAGCAAGGGTGCATGGG

TGAATGGTCTCCGAGTGGATCTCCCAGCTACTGTGTTAACCTCTGTGTCTGTGAGGA

AGATGCCAGACGGCTCCATGTTTGTCCACCAGAAGGCCGGGGTCCAGGTGTGGCTTG

GAAAGGATGGGCATCTGGATGTGATGGTTGGTGATGACCATGCAGCCCGTGCTGTGT

GGGGTCTGTGGAAACTTTGAT

Translated Protein

STAAVKPWRTDSFCPLQCPAHSHYSVCTRSCQGSCAALSGLTGCTTRCFEGCECDDHFL

LSQGVCIPAQDCGCVHNGQYVPVNSSLMSSDCSELCYCSETTGLTCRAAGCPSGQVCEI

QAGVRQCRAAHGLCSLSVGANLTTFDGALNTISSPGVYELSSRCPGFQKNVPWYRLLA

DVQPCLNNDKIVSKVHIFFQDGMVTVIPSKGAWVNGLRVDLPATVLTSVSVRKMPDGS

MFVHQKAGVQVWLGKDGHLDVMVGDDHAARAVWGLWKL

>pGAD10/B4.15

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GAATGGAACCACGTGCAAAGCTTTCTGCAAAGACGGCTGCAGAAACGGAGGTGCCT

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CAGACATTGATGAGTGCTCTGAGGGCTTTGTTCAGTGTGACAGCCGTGCCAACTGCA

TTAACCTGCCTGGGTGGTACCACTGTGAGTGCAGAGATGGCTACCATGACAATGGG

ATGTTTGCGCCAGGTGGAGAATCCTGTGAAGATATTGATGAATGTGGGA

Translated Protein

FHGLVQKIMELQDILSKTSAKLSRAEQRMNRLDQCYCERTCTMKGATYREFESWTDGC

KNCTCLNGTIQCETLVCPAPDCPAKSAPAYVDGKCCKECKSTCQFQGRSYFEGERSTVF

SASGMCVLYECKDQTMKLVENAGCPALDCPESHQIALSHSCCKVCKGYDFCSEKHTCM

ENSVCRNLNDRAVCSCRDGFRALREDNAYCEDIDECAEGRHYCRENTMCVNTPGSFLCI

CQTGYIRIDDYSCTEHDECLTNQHNCDENALCFNTVGGHNCVCKPGYTGNGTTCKAFC

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ACCACGTGCAAAGCTTTCTGCAAAGACGGCTGCAGAAACGGAGGTGCCTGCATTGC

TGCCAATGTCTGTGCTTGCCCACAAGGCTTCACCGGACCCAGCTGTGAGACAGACAT

TGATGAGTGCTCTGAGGGCTTTGTTCAGTGTGACAGCCGTGCCAACTGCATTAACCT

GCCTGGGTGGTACCACTGTGAGTGCAGAGATGGCTACCATGACAATGGGATGTTTGC

GCCAGGTGGAGAATCCTGTGAAGATATTGATGAATGTGGGACTGGGAGGCACAGCT

GTGCCAATGACACCATTTGCTTCAACTTGGACGGTGGCTACGATTGCCGGTGTCCCC

ATGGAAAGAACTGCACAGGGGACTGCGTGCACGACGGGAAAGTCAAACACAACGG

CCAGATCTGGGTGCTGGAGAACGACAGGTGCTCTGTGTGTTCCTGCCAGACTGGATT

TGTTATGTGTCGACGGATGGTCTGTGACTGCGAAAACCCCACAGTTGACCTCTCCTG

CTGCCCTGAGTGCGACCCAAGGCTGAGCAGCCAGTGCCTGCATCAAAACGGGGAAA

CCGTGTACAACAGCGGTGACACCTGGGTCCAGGATTGCCGTCAGTGCCGCTGCTTGC

AAGGAGAAGTTGACTGCTGGCCCCTGGCTTGCCCAGAGGTAGAGTGTGAATTTAGT

GTCCTTCCTGAGAACGAGTGCTGCCCACGCTGTGTCACCGATCCTTGTCAGGCTGAC

Translated Protein

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SCCKVCKGYDFCSEKHTCMENSVCRNLNDRAVCSCRDGFRALREDNAYCEDIDECAEG

RHYCRENTMCVNTPGSFLCICQTGYIRIDDYSCTEHDECLTNQHNCDENALCFNTVGGH

NCVCKPGYTGNGTTCKAFCKDGCRNGGACIAANVCACPQGFTGPSCETDIDECSEGFV

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Translated Protein

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ATGTTTGCGCCAGGTGGAGAATCCTGTGAAGATATTGATGAATGTGGGACTGGG

Translated Protein

FHGLVQKIMELQDILSKTSAKLSRAEQRMNRLDQCYCERTCTMKGATYREFESWTDGC

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CQTGYIRIDDYSCTEHDECLTNQHNCDENALCFNTVGGHNCVCKPGYTGNGTTCKAFC

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TTGACCTCTCCTGCTGCCCTGAGTGCGACCCAAGGCTGAGCAGCCAGTGCCTGCATC

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GTCAGGCTGACACCATCCGCAATGACATCACCAAAACCTGCCTGGACGAGATGAAC

GTGGTTCGCTTCACTGGGTCTTCCTGGATCAAGCACGGCACGGAGTGCACCCTCTGC

CAGTGCAAGAACGGCCACGTGTGCTGCTCAGTGGACCCACAGTGCCTCCAGGAGCT

GTGAAGTTAACTGCCTCATGGGAGATACCTGTTCAAAGAATGATTTCTCATTTAAAA

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CCAGCGAGGTTCCTCACTGAAGCCATGGAATGAAAGGCAGTGAGTGAGCTATATTTT

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ATTGTAATGATAAAATACTGCACAGGCATGGTTATGTAACATTTTCTAACCGG

Translated Protein

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ACACCATGTGTGTGAACACACCGGGCTCTTTCCTGTGTATCTGCCAAACAGGGTACA

TCAGAATCGACGATTACTCGTGTACGGAACATGACGAGTGCCTCACAAACCAGCAC

AACTGTGACGAGAACGCTTTGTGCTTTAACACCGTTGGAGGTCACAACTGCGTCTGC

AAGCCTGGCTACACTGGGAATGGAACCACGTGCAAAGCTTTCTGCAAAGACGGCTG

CAGAAACGGAGGTGCCTGCATTGCTGCCAATGTCTGTGCTTGCCCACAAGGCTTCAC

CGGACCCAGCTGTGAGACAGACATTGATGAGTGCTCTGAGGGCTTTGTTCAGTGTGA

CAGCCGTGCCAACTGCATTAACCTGCCTGGGTGGTACCACTGTGAGTGCAGAGATGG

CTACCATGACAATGGGATGTTTGCGCCAGGTGGAGAATCCTGTGAAGATATTGATGA

ATGTGGGACTGGGAGGCACAGCTGTGCCAATGACACCATTTGCTTCAACTTGGACGG

TGGCTACGATTGCCGGTGTCCCCATGGAAAGAACTGCACAGGGGACTGCGTGCACG

ACGGGAAAGTCAAACACAACGGCCAGATCTGGGTGCTGGAGAACGACAGGTGCTCT

GTGTGTTCCTGCCAGACTGGATTTGTTATGTGTCGACGGATGGTCTGTGACTGCGAA

AACCCCACAGTTGACCTCTCCTGCTGCCCTGAGTGCGACCCAAGGCTGAGCAGCCAG

TGCCTGCATCAAAACGGGGAAACCGTGTACAACAGCGGTGACACCTGGGTCCAGGA

TTGCCGTCAGTGCCGCTGCTTGCAAGGAGAAGTTGACTGCTGGCCCCTGGCTTGCCC

AGAGGTAGAGTGTGAATTTAGTGTCCTTCCTGAGAACGAGTGCTGCCCACGCTGTGT

CACCGATCCTTGTCAGGCTGACACCATCCGCAATGACATCACCAAAACCTGCCTGGA

CGAGATGAACGTGGTTCGCTTCACTGGGTCTTCCTGGATCAAGCACGGCACGGAGTG

CACCCTCTGCCAGTGCAAGAACGGCCACGTGTGCTGCTCAGTGGACCCACAGTGCCT

CCAGGAGCTGTGAAGTTAACTGCCTCATGGGAGATACCTGTTCAAAGAATGATTTCT

CATTTAAAAAGACCAAAAAAACAAAAAAGAAAAAAAGTGATGTGCGGCCAGCCAA

ATGCAACTGTGTCAATGGCTGGGCAGACTGATGGCGATTACGGCTCTGTAGAGCTTT

GAGGAACATCACTGAGGAAACCAGATGGCAGTTCCGCCTTTACTGTTCCTGGGATCA

CCTTATGGAGGAATGGCTGTGAATCACAGGCCTTGACATCCCCAGCCCTGGAGAAG

AAGCCTGAGCCCATCAGCTCTGGGGAAGTCTCTCCCTCTCTCCCTCCCTCCACAGGC

ACAGGACATGTCTAG

Translated Protein

NDRAVCSCRDGFRALREDNAYCEDIDECAEGRHYCRENTMCVNTPGSFLCICQTGYIRI

DDYSCTEHDECLTNQHNCDENALCFNTVGGHNCVCKPGYTGNGTTCKAFCKDGCRNG

GACIAANVCACPQGFTGPSCETDIDECSEGFVQCDSRANCINLPGWYHCECRDGYHDNG

MFAPGGESCEDIDECGTGRHSCANDTICFNLDGGYDCRCPHGKNCTGDCVHDGKVKHN

GQIWVLENDRCSVCSCQTGFVMCRRMVCDCENPTVDLSCCPECDPRLSSQCLHQNGET

VYNSGDTWVQDCRQCRCLQGEVDCWPLACPEVECEFSVLPENECCPRCVTDPCQADTI

RNDITKTCLDEMNVVRFTGSSWIKHGTECTLCQCKNGHVCCSVDPQCLQEL

>pGAD10/B4.84

GAAGGGAACCACCTACCGGGAGTTCGAGTCCTGGACAGACGGCTGCAAGAACTGCA

CATGCTTGAATGGGACCATCCAGTGCGAGACTCTGGTCTGCCCTGCTCCCGACTGCC

CGGCTAAATCGGCTCCAGCGTACGTGGATGGCAAGTGCTGTAAGGAGTGCAAGTCC

ACCTGCCAGTTCCAGGGGCGGAGCTACTTTGAGGGAGAAAGGAGCACAGTCTTCTC

AGCTTCCGGAATGTGCGTCTTGTATGAATGCAAGGATCAGACCATGAAGCTTGTTGA

GAACGCCGGCTGCCCGGCTTTAGATTGCCCCGAGTCTCATCAGATCGCCTTGTCTCA

CAGCTGCTGCAAGGTTTGCAAAGGTTATGACTTCTGTTCTGAGAAGCATACATGCAT

GGAGAACTCAGTCTGCAGGAACCTGAACGACAGGGCAGTGTGCAGCTGCCGGGATG

GTTTCCGGGCCCTCCGGGAGGACAATGCCTACTGTGAAGACATTGACGAGTGTGCA

GAGGGGCGCCATTACTGCCGTGAGAACACCATGTGTGTGAACACACCGGGCTCTTTC

CTGTGTATCTGCCAAACAGGGTACATCAGAATCGACGATTACTCGTGTACGGAACAT

GACGAGTGCCTCACAAACCAGCACAACTGTGACGAGAACGCTTTGTGCTTTAACAC

CGTTGGAGGTCACAACTGCGTCTGCAAGCCTGGCTACACTGGGAATGGAACCACGT

GCAAAGCTTTCTGCAAAGACGGCTGCAGAAACGGAGGTGCCTGCATTGCTGCCAAT

GTCTGTGCTTGCCCACAAGGCTTCACCGGACCCAGCTGTGAGACAGACATTGATGAG

TGCTCTGAGGGCTTTGTTCAGTGTGACAGCCGTGCCAACTGCATTAACCTGCCTGGG

TGGTACCACTGTGAGTGCAGAGATGGCTACCATGACAATGGGATGTTTGCGCCAGGT

GGAGAATCCTGTGAAGATATTGATGAATGTGGGACTGGGAGGCACAGCTGTGCCAA

TGACACCATTTGCTTCAACTTGGACGGTGGCTACGATTGCCGGTGTCCCCATGGAAA

GAACTGCACAGGGGACTGCGTGCACGACGGGAAAGTCAAACACAACGGCCAGATCT

GGGTGCTGGAGAACGACAGGTGCTCTGTGTGTTCCTGCCAGACTGGATTTGTTATGT

GTCGACGGATGGTCTGTGACTGCGAAAACCCCACAGTTGACCTCTCCTGCTGCCCTG

AGTGCGACCCAAGGCTGAGCAGCCAGTGCCTGCATCAAAACGGGGAAACCGTGTAC

AACAGCGGTGACACCTGGGTCCAGGATTGCCGTCAGTGCCGCTGCTTGCAAGGAGA

AGTTGACTGCTGGCCCCTGGCTTGCCCAGAGGTAGAGTGTGAATTTAGTGTCCTTCC

TGAGAACGAGTGCTGCCCACGCTGTGTCACCGATCCTTGTCAGGCTGACACCATCCG

CAATGACATCACCAAAACCTGCCTGGACGAGATGAACGTGGTTCGCTTCACTGGGTC

TTCCTGGATCAAGCACGGCACGGAGTGCACCCTCTGCCAGTGCAAGAACGGCCACG

TGTGCTGCTCAGTGGACCCACAGTGCCTCCAGGAGCTGTGAAGTTAACTGCCTCATG

GGAGATACCTGTTCAAAGAATGATTTCTCATTTAAAAAGACTAAAAAAAAAAAAAA

AAAAAAAAA

Translated Protein

KGTTYREFESWTDGCKNCTCLNGTIQCETLVCPAPDCPAKSAPAYVDGKCCKECKSTC

QFQGRSYFEGERSTVFSASGMCVLYECKDQTMKLVENAGCPALDCPESHQIALSHSCCK

VCKGYDFCSEKHTCMENSVCRNLNDRAVCSCRDGFRALREDNAYCEDIDECAEGRHY

CRENTMCVNTPGSFLCICQTGYIRIDDYSCTEHDECLTNQHNCDENALCFNTVGGHNCV

CKPGYTGNGTTCKAFCKDGCRNGGACIAANVCACPQGFTGPSCETDIDECSEGFVQCDS

RANCINLPGWYHCECRDGYHDNGMFAPGGESCEDIDECGTGRHSCANDTICFNLDGGY

DCRCPHGKNCTGDCVHDGKVKHNGQIWVLENDRCSVCSCQTGFVMCRRMVCDCENP

TVDLSCCPECDPRLSSQCLHQNGETVYNSGDTWVQDCRQCRCLQGEVDCWPLACPEVE

CEFSVLPENECCPRCVTDPCQADTIRNDITKTCLDEMNVVRFTGSSWIKHGTECTLCQCK

NGHVCCSVDPQCLQEL

Figure 7. GenBank Rat Nell2 and Cloned Rat Nell2 DNA Sequence Alignment.

DNA sequence alignment of the rat Nell2 identified by S. Kuroda and submitted in GenBank (U48245, Kuroda et al., 1999) with the cloned Rat Nell2 generated by RT-PCR. The sequence alignment was done on ClustalX (Gap Opening 10, Gap Extension 0.20 and Scoring Matrix Gonnet 250, Thompson et al., 1997) with further analysis on GeneDoc (Nicholas, 1997). Ten nucleic acid differences result in seven amino acid changes between the two sequences (**Figure 10**).

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Nell2  :  ------------------------------------------------  :    -
U48245 :  GTCCTTTCTCTCGCCGGGTTTGGAGACACGCTCCCGATTTCGAGGGGAG  :   49

Nell2  :  ----------------------------ATGGAATCCCGGGTATTACT  :   20
U48245 :  GGAGACGATGGACTGAGACGATGCACGCCATGGAATCCCGGGTATTACT  :   98
                                      ATGGAATCCCGGGTATTACT

Nell2  :  GAGAACGTTCTGCGTGATCCTCGGGCTCGAAGCGGTTTGGGGACTTGGT  :   69
U48245 :  GAGAACGTTCTGCGTGATCCTCGGGCTCGAAGCGGTTTGGGGACTTGGT  :  147
          GAGAACGTTCTGCGTGATCCTCGGGCTCGAAGCGGTTTGGGGACTTGGT

Nell2  :  GTGGACCCCTCCCTACAGATTGACGTCTTATCAGAGTTAGAACTTGGGG  :  118
U48245 :  GTGGACCCCTCCCTACAGATTGACGTCTTATCAGAGTTAGAACTTGGGG  :  196
          GTGGACCCCTCCCTACAGATTGACGTCTTATCAGAGTTAGAACTTGGGG

Nell2  :  AGTCCACAGCTGGAGTGCGCCAAGTCTCAGGACTGCATAATGGGACGAA  :  167
U48245 :  AGTCCACAGCTGGAGTGCGCCAAGTCCCAGGACTGCATAATGGGACGAA  :  245
          AGTCCACAGCTGGAGTGCGCCAAGTC CAGGACTGCATAATGGGACGAA

Nell2  :  AGCCTTCCTCTTCCAAGATTCCCCCAGAAGCATAAAAGCACCCATTGCT  :  216
U48245 :  AGCCTTCCTCTTCCAAGATTCCCCCAGAAGCATAAAAGCACCCATTGCT  :  294
          AGCCTTCCTCTTCCAAGATTCCCCCAGAAGCATAAAAGCACCCATTGCT

Nell2  :  ACAGCTGAGCGGTTTTTCCAGAAGCTGAGGAATAAACACGAGTTCACAA  :  265
U48245 :  ACAGCTGAGCGGTTTTTCCAGAAGCTGAGGAATAAACACGAGTTCACAA  :  343
          ACAGCTGAGCGGTTTTTCCAGAAGCTGAGGAATAAACACGAGTTCACAA

Nell2  :  TTCTGGTGACCCTGAAACAGATCCACTTAAATTCGGAAGTCATTCTCTC  :  314
U48245 :  TTCTGGTGACCCTGAAACAGATCCACTTAAATTCGGGAGTCATTCTCTC  :  392
          TTCTGGTGACCCTGAAACAGATCCACTTAAATTCGG AGTCATTCTCTC

Nell2  :  CATCCACCACTTGGATCACAGGTACCTGGAACTGGAAAGCAGCGGCCAC  :  363
U48245 :  CATCCACCACTTGGATCACAGGTACCTGGAACTGGAAAGCAGCGGCCAC  :  441
          CATCCACCACTTGGATCACAGGTACCTGGAACTGGAAAGCAGCGGCCAC

Nell2  :  CGGAATGAGATCAGACTGCATTACCGCTCTGGAACTCACCGCCCGCACA  :  412
U48245 :  CGGAATGAGATCAGACTGCATTACCGCTCTGGAACTCACCGCCCGCACA  :  490
          CGGAATGAGATCAGACTGCATTACCGCTCTGGAACTCACCGCCCGCACA
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Nell2  : CGGAAGTGTTTCCTTATATTTTGGCTGATGCCAAGTGGCACAAGCTCTC : 461
U48245 : CGGAAGTGTTTCCTTATATTTTGGCTGATGCCAAGTGGCACAAGCTCTC : 539
         CGGAAGTGTTTCCTTATATTTTGGCTGATGCCAAGTGGCACAAGCTCTC

Nell2  : CTTAGCCTTCAGTGCCTCCCACTTAATTTTACACATCGACTGCAACAAG : 510
U48245 : CTTAGCCTTCAGTGCCTCCCACTTAATTTTACACATCGACTGCAACAAG : 588
         CTTAGCCTTCAGTGCCTCCCACTTAATTTTACACATCGACTGCAACAAG

Nell2  : ATCTATGAACGAGTGGTGGAAATGCCTTCTACAGACTTGCCTCTGGGCA : 559
U48245 : ATCTATGAACGAGTGGTGGAAATGCCTTCTACAGACTTGCCTCTGGGCA : 637
         ATCTATGAACGAGTGGTGGAAATGCCTTCTACAGACTTGCCTCTGGGCA

Nell2  : CCACATTTTGGTTGGGACAGAGAAATAACGCACACGGGTATTTTAAGGG : 608
U48245 : CCACATTTTGGTTGGGACAGAGAAATAACGCACACGGGTATTTTAAGGG : 686
         CCACATTTTGGTTGGGACAGAGAAATAACGCACACGGGTATTTTAAGGG

Nell2  : AATAATGCAAGATGTGCAATTACTTGTCATGCCCCAGGGGTTCATCGCT : 657
U48245 : AATAATGCAAGATGTGCAATTACTTGTCATGCCCCAGGGGTTCATCGCT : 735
         AATAATGCAAGATGTGCAATTACTTGTCATGCCCCAGGGGTTCATCGCT

Nell2  : CAGTGCCCGGATCTTAATCGAACCTGTCCAACATGCAACGACTTCCATG : 706
U48245 : CAGTGCCCGGATCTTAATCGAACCTGTCCAACATGCAACGACTTCCATG : 784
         CAGTGCCCGGATCTTAATCGAACCTGTCCAACATGCAACGACTTCCATG

Nell2  : GGCTTGTGCAGAAAATCATGGAGCTGCAGGACATTTTATCGAAGACGTC : 755
U48245 : GGCTTGTGCAGAAAATCATGGAGCTGCAGGACATTTTATCGAAGACGTC : 833
         GGCTTGTGCAGAAAATCATGGAGCTGCAGGACATTTTATCGAAGACGTC

Nell2  : AGCCAAGTTGTCTAGAGCTGAACAACGAATGAACAGGCTGGATCAGTGC : 804
U48245 : AGCCAAGTTGTCTAGAGCTGAACAACGAATGAACAGGCTGGATCAGTGC : 882
         AGCCAAGTTGTCTAGAGCTGAACAACGAATGAACAGGCTGGATCAGTGC

Nell2  : TACTGTGAGCGGACGTGCACCATGAAGGGAGCCACCTACCGGGAGTTCG : 853
U48245 : TACTGTGAGCGGACGTGCACCATGAAGGGAGCCACCTACCGGGAGTTCG : 931
         TACTGTGAGCGGACGTGCACCATGAAGGGAGCCACCTACCGGGAGTTCG

Nell2  : AGTCCTGGACAGACGGCTGCAAGAACTGCACATGCTTGAATGGGACCAT : 902
U48245 : AGTCCTGGACAGACGGCTGCAAGAACTGCACATGCTTGAATGGGACCAT : 980
         AGTCCTGGACAGACGGCTGCAAGAACTGCACATGCTTGAATGGGACCAT
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Nell2  : CCAGTGCGAGACTCTGGTCTGCCCTGCTCCCGACTGCCCGGCTAAATCG :  951
U48245 : CCAGTGCGAGACTCTGGTCTGCCCTGCTCCCGACTGCCCGGCTAAATCG : 1029
         CCAGTGCGAGACTCTGGTCTGCCCTGCTCCCGACTGCCCGGCTAAATCG

Nell2  : GCTCCAGCGTACGTGGATGGCAAGTGCTGTAAGGAGTGCAAGTCCACCT : 1000
U48245 : GCTCCAGCGTACGTGGATGGCAAGTGCTGTAAGGAGTGCAAGTCCACCT : 1078
         GCTCCAGCGTACGTGGATGGCAAGTGCTGTAAGGAGTGCAAGTCCACCT

Nell2  : GCCAGTTCCAGGGGCGGAGCTACTTTGAGGGAGAAAGGAGCACAGTCTT : 1049
U48245 : GCCAGTTCCAGGGGCGGAGCTACTTTGAGGGAGAAAGGAGCACAGTCTT : 1127
         GCCAGTTCCAGGGGCGGAGCTACTTTGAGGGAGAAAGGAGCACAGTCTT

Nell2  : CTCAGCTTCCGGAATGTGCGTCTTGTATGAATGCAAGGATCAGACCATG : 1098
U48245 : CTCAGCTTCCGGAATGTGCGTCTTGTATGAATGCAAGGATCAGACCATG : 1176
         CTCAGCTTCCGGAATGTGCGTCTTGTATGAATGCAAGGATCAGACCATG

Nell2  : AAGCTTGTTGAGAACGCCGGCTGCCCGGCTTTAGATTGCCCCGAGTCTC : 1147
U48245 : AAGCTTGTTGAGAACGCCGGCTGCCCGGCTTTAGATTGCCCCGAGTCTC : 1225
         AAGCTTGTTGAGAACGCCGGCTGCCCGGCTTTAGATTGCCCCGAGTCTC

Nell2  : ATCAGATCGCCTTGTCTCACAGCTGCTGCAAGGTTTGCAAAGGTTATGA : 1196
U48245 : ATCAGATCGCCTTGTCTCACAGCTGCTGCAAGGTTTGCAAAGGTTATGA : 1274
         ATCAGATCGCCTTGTCTCACAGCTGCTGCAAGGTTTGCAAAGGTTATGA

Nell2  : CTTCTGTTCTGAGAAGCATACATGCACGGAGAACTCAGTCTGCAGGAAC : 1245
U48245 : CTTCTGTTCTGAGAAGCATACATGCATGGAGAACTCAGTCTGCAGGAAC : 1323
         CTTCTGTTCTGAGAAGCATACATGCA GGAGAACTCAGTCTGCAGGAAC

Nell2  : CTGAACGACAGGGCAGTGTGCAGCTGCCGGGATGGTTTCCGGGCCCTCC : 1294
U48245 : CTGAACGACAGGGCAGTGTGCAGCTGCCGGGATGGTTTCCGGGCCCTCC : 1372
         CTGAACGACAGGGCAGTGTGCAGCTGCCGGGATGGTTTCCGGGCCCTCC

Nell2  : GGGAGGACAATGCCTACTGTGAAGACATTGACGAGTGTGCAGAGGGGCG : 1343
U48245 : GGGAGGACAATGCCTACTGTGAAGACATTGACGAGTGTGCAGAGGGGCG : 1421
         GGGAGGACAATGCCTACTGTGAAGACATTGACGAGTGTGCAGAGGGGCG

Nell2  : CCATTACTGCCGTGAGAACACCATGTGTGTGAACACACCGGGCTCTTTC : 1392
U48245 : CCATTACTGCCGTGAGAACACCATGTGTGTGAACACACCGGGCTCTTTC : 1470
         CCATTACTGCCGTGAGAACACCATGTGTGTGAACACACCGGGCTCTTTC
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Nell2  :  CTGTGTATCTGCCAAACAGGGTACATCAGAATCGACGATTACTCGTGTA  : 1441
U48245 :  CTGTGTATCTGCCAAACAGGGTACATCAGAATCGACGATTACTCGTGTA  : 1519
          CTGTGTATCTGCCAAACAGGGTACATCAGAATCGACGATTACTCGTGTA

Nell2  :  CGGAACATGACGAGTGCCTCACAAACCAGCACAACTGTGACGAGAACGC  : 1490
U48245 :  CGGAACATGACGAGTGCCTCACAAACCAGCACAACTGTGACGAGAACGC  : 1568
          CGGAACATGACGAGTGCCTCACAAACCAGCACAACTGTGACGAGAACGC

Nell2  :  TTTGTGCTTTAACACCGTTGGAGGTCACAACTGCGTCTGCAAGCCTGGC  : 1539
U48245 :  TTTGTGCTTTAACACCGTTGGAGGTCACAACTGCGTCTGCAAGCCTGGC  : 1617
          TTTGTGCTTTAACACCGTTGGAGGTCACAACTGCGTCTGCAAGCCTGG

Nell2  :  TACACTGGGAATGGAACCACGTGCAAAGCTTTCTGCAAAGACGGCTGCA  : 1588
U48245 :  TACACTGGGAATGGAACCACGTGCAAAGCTTTCTGCAAAGACGGCTGCA  : 1666
          TACACTGGGAATGGAACCACGTGCAAAGCTTTCTGCAAAGACGGCTGCA

Nell2  :  CAAACGAAGGTGCCTGCATTGCTGCCAATGTCTGTGCTTGCCCACAAGG  : 1637
U48245 :  AAAACGCAGGTGCCTGCATTGCTGCCAATGTCTGTGCTTGCCCACAAGG  : 1715
           AAACG AGGTGCCTGCATTGCTGCCAATGTCTGTGCTTGCCCACAAGG

Nell2  :  CTTCACCGGACCCAGCTGTGAGACAGACATTGATGAGTGCTCTGAGGGC  : 1686
U48245 :  CTTCACCGGACCCAGCTGTGAGACAGACATTGATGAGTGCTCTGAGGGC  : 1764
          CTTCACCGGACCCAGCTGTGAGACAGACATTGATGAGTGCTCTGAGGGC

Nell2  :  TTTGTTCAGTGTGACAGCCGTGCCAACTGCATTAACCTGCCTGGGTGGT  : 1735
U48245 :  TTTGTTCAGTGTGACAGCCGTGCCAACTGCATTAACCTGCCTGGGTGGT  : 1813
          TTTGTTCAGTGTGACAGCCGTGCCAACTGCATTAACCTGCCTGGGTGGT

Nell2  :  ACCACTGTGAGTGCAGAGATGGCTACCATGACAATGGGATGTTTGCGCC  : 1784
U48245 :  ACCACTGTGAGTGCAGAGATGGCTACCATGACAATGGGATGTTTGCGCC  : 1862
          ACCACTGTGAGTGCAGAGATGGCTACCATGACAATGGGATGTTTGCGCC

Nell2  :  AGGTGGAGAATCCTGTGAAGATATTGATGAATGTGGGACTGGGAGGCAC  : 1833
U48245 :  AGGTGGAGAATCCTGTGAAGATATTGATGAATGTGGGACTGGGAGGCAC  : 1911
          AGGTGGAGAATCCTGTGAAGATATTGATGAATGTGGGACTGGGAGGCAC

Nell2  :  AGCTGTGCCAATGACACCATTTGCTTCAACTTGGACGGTGGCTACGATT  : 1882
U48245 :  AGCTGTGCCAATGACACCATTTGCTTCAACTTGGACGGTGGCTACGATT  : 1960
          AGCTGTGCCAATGACACCATTTGCTTCAACTTGGACGGTGGCTACGATT
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Nell2  : GCCGGTGTCCCCATGGAAAGAACTGCACAGGGGACTGCGTGCACGACGG : 1931
U48245 : GCCGGTGTCCCCATGGAAAGAACTGCACAGGGGACTGCGTGCACGACGG : 2009
         GCCGGTGTCCCCATGGAAAGAACTGCACAGGGGACTGCGTGCACGACGG

Nell2  : GAAAGTCAAACACAACGGCCAGATCTGGGTGCTGGAGAACGACAGGTGC : 1980
U48245 : GAAAGTCAAACACAACGGCCAGATCTGGGTGCTGGAGAACGACAGGTGC : 2058
         GAAAGTCAAACACAACGGCCAGATCTGGGTGCTGGAGAACGACAGGTGC

Nell2  : TCTGTGTGTTCCTGCCAGACTGGATTTGTTATGTGTCCACGGATGGTCT : 2029
U48245 : TCTGTGTGTTCCTGCCAGACTGGATTTGTTATGTGCCACGGATGGTCT : 2107
         TCTGTGTGTTCCTGCCAGACTGGATTTGTTATGTG C ACGGATGGTCT

Nell2  : GTGACTGCGAAAACCCCACAGTTGACCTCTCCTGCTGCCCTGAGTGCGA : 2078
U48245 : GTGACTGCGAAAACCCCACAGTTGACCTCTCCTGCTGCCCTGAGTGCGA : 2156
         GTGACTGCGAAAACCCCACAGTTGACCTCTCCTGCTGCCCTGAGTGCGA

Nell2  : CCCAAGGCTGAGCAGCCAGTGCCTGCATCAAAACGGGGAAACCGTGTAC : 2127
U48245 : CCCAAGGCTGAGCAGCCAGTGCCTGCATCAAAACGGGGAAACCGTGTAC : 2205
         CCCAAGGCTGAGCAGCCAGTGCCTGCATCAAAACGGGGAAACCGTGTAC

Nell2  : AACAGCGGTGACACCTGGGTCCAGGATTGCCGTCAGTGCCGCTGCTTGC : 2176
U48245 : AACAGCGGTGACACCTGGGCCAGGATTGCCGTCAGTGCCGCTGCTTGC : 2254
         AACAGCGGTGACACCTGGG CCAGGATTGCCGTCAGTGCCGCTGCTTGC

Nell2  : AAGCAGAAGTTGACTGCTGGCCCCTGGCTTGCCCAGAGGTAGAGTGTGA : 2225
U48245 : AAGAAGAAGTTGACTGCTGGCCCCTGGCTTGCCCAGAGGTAGAGTGTGA : 2303
         AAG AGAAGTTGACTGCTGGCCCCTGGCTTGCCCAGAGGTAGAGTGTGA

Nell2  : ATTTAGTGTCCTTCCTGAGAACGAGTGCTGCCCACGCTGTGTCACCGAT : 2274
U48245 : ATTTAGTGTCCTTCCTGAGAACGAGTGCTGCCCACGCTGTGTCACCGAT : 2352
         ATTTAGTGTCCTTCCTGAGAACGAGTGCTGCCCACGCTGTGTCACCGAT

Nell2  : CCTTGTCAGGCTGACACCATCCGCAATGACATCACCAAAACCTGCCTGG : 2323
U48245 : CCTTGTCAGGCTGACACCATCCGCAATGACATCACCAAAACCTGCCTGG : 2401
         CCTTGTCAGGCTGACACCATCCGCAATGACATCACCAAAACCTGCCTGG

Nell2  : ACGAGATGAACGTGGTTCGCTTCACTGGGTCTTCCTGGATCAAGCACGG : 2372
U48245 : ACGAGATGAACGTGGTTCGCTTCACTGGGTCTTCCTGGATCAAGCACGG : 2450
         ACGAGATGAACGTGGTTCGCTTCACTGGGTCTTCCTGGATCAAGCACGG
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Nell2  : CACGGAGTGCACCCTCTGCCAGTGCAAGAACGGCCACGTGTGCTGCTCA : 2421
U48245 : CACGGAGTGCACCCTCTGCCAGTGCAAGAACGGCCACGTGTGCTGCTCA : 2499
         CACGGAGTGCACCCTCTGCCAGTGCAAGAACGGCCACGTGTGCTGCTCA

Nell2  : GTGGACCCACAGTGCCTCCAGGAGCTGTGA------------------- : 2451
U48245 : GTGGACCCACAGTGCCTCCAGGAGCTGTGAAGTTAACTGCCTCATGGGA : 2548
         GTGGACCCACAGTGCCTCCAGGAGCTGTGA

Nell2  : ------------------------------------------------ :    -
U48245 : GATACCTGTTAAAGAATGATTTCTCATTTAAAAAAAAAAAAAAAAAAAA : 2597

Nell2  : ---------------------- :    -
U48245 : AAAAAAAAAAAAAAAAAAAAAA : 2618
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Figure 8. Nel Family Protein Sequences.

Nel family members have been identified in rat, human, chicken and mouse, and the protein sequences are listed here (**Table 9**). The Nel family was found to be highly conserved at the amino acid level (**Figure 14**). rN2* Rat Cloned Nell2, rN2 Rat Protein Kinase C Binding Protein Nell2 (NP_112332), cN2 Chicken Nel-like 2 Homolog (NP_058023), mN2 Mouse Mel (AAB02924), hN2 Human Nel-like 2 (NP_006150), hN Human Cerebral Protein-12 (BAB46925), cNp Chicken Nel Protein Precursor (Q90827), cN Chicken Nel Protein (JP0076), cN1 Chicken Nel-like 1 (NP_006148), rN1 Rat Protein Kinase C Binding Protein Nell1 (AAC72252), hN1 Human Nel-related Protein (BAA11680).

>rN2*

MESRVLLRTFCVILGLEAVWGLGVDPSLQIDVLSELELGESTAGVRQVSGLHNGTKAFL

FQDSPRSIKAPIATAERFFQKLRNKHEFTILVTLKQIHLNSEVILSIHHLDHRYLELESSGH

RNEIRLHYRSGTHRPHTEVFPYILADAKWHKLSLAFSASHLILHIDCNKIYERVVEMPST

DLPLGTTFWLGQRNNAHGYFKGIMQDVQLLVMPQGFIAQCPDLNRTCPTCNDFHGLVQ

KIMELQDILSKTSAKLSRAEQRMNRLDQCYCERTCTMKGATYREFESWTDGCKNCTCL

NGTIQCETLVCPAPDCPAKSAPAYVDGKCCKECKSTCQFQGRSYFEGERSTVFSASGMC

VLYECKDQTMKLVENAGCPALDCPESHQIALSHSCCKVCKGYDFCSEKHTCTENSVCR

NLNDRAVCSCRDGFRALREDNAYCEDIDECAEGRHYCRENTMCVNTPGSFLCICQTGYI

RIDDYSCTEHDECLTNQHNCDENALCFNTVGGHNCVCKPGYTGNGTTCKAFCKDGCR

NEGACIAANVCACPQGFTGPSCETDIDECSEGFVQCDSRANCINLPGWYHCECRDGYHD

NGMFAPGGESCEDIDECGTGRHSCANDTICFNLDGGYDCRCPHGKNCTGDCVHDGKVK

HNGQIWVLENDRCSVCSCQTGFVMCRRMVCDCENPTVDLSCCPECDPRLSSQCLHQNG

ETVYNSGDTWVQDCRQCRCLQGEVDCWPLACPEVECEFSVLPENECCPRCVTDPCQAD

TIRNDITKTCLDEMNVVRFTGSSWIKHGTECTLCQCKNGHVCCSVDPQCLQEL

>rN2

MESRVLLRTFCVILGLEAVWGLGVDPSLQIDVLSELELGESTAGVRQVPGLHNGTKAFL

FQDSPRSIKAPIATAERFFQKLRNKHEFTILVTLKQIHLNSGVILSIHHLDHRYLELESSGH

RNEIRLHYRSGTHRPHTEVFPYILADAKWHKLSLAFSASHLILHIDCNKIYERVVEMPST

DLPLGTTFWLGQRNNAHGYFKGIMQDVQLLVMPQGFIAQCPDLNRTCPTCNDFHGLVQ

KIMELQDILSKTSAKLSRAEQRMNRLDQCYCERTCTMKGATYREFESWTDGCKNCTCL

NGTIQCETLVCPAPDCPAKSAPAYVDGKCCKECKSTCQFQGRSYFEGERSTVFSASGMC

VLYECKDQTMKLVENAGCPALDCPESHQIALSHSCCKVCKGYDFCSEKHTCMENSVCR

NLNDRAVCSCRDGFRALREDNAYCEDIDECAEGRHYCRENTMCVNTPGSFLCICQTGYI

RIDDYSCTEHDECLTNQHNCDENALCFNTVGGHNCVCKPGYTGNGTTCKAFCKDGCK

NGGACIAANVCACPQGFTGPSCETDIDECSEGFVQCDSRANCINLPGWYHCECRDGYHD

NGMFAPGGESCEDIDECGTGRHSCANDTICFNLDGGYDCRCPHGKNCTGDCVHDGKVK

HNGQIWVLENDRCSVCSCQTGFVMCQRMVCDCENPTVDLSCCPECDPRLSSQCLHQNG

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WTHSCQQCRCLEGEVDCWPLTCPNLSCEYTAILEGECCPRCVSDPCLADNITYDIRKTCL

DSYGVSRLSGSVWTMAGSPCTTCKCKNGRVCCSVDFECLQNN

Figure 9. Publications.

K. G. Sutton, J. McRory, **H. Guthrie**, T. H. Murphy and T. P. Snutch (1999). P/Q-type Ca^{2+} channels mediate the activity-dependent feedback of syntaxin-1A. Nature 401:800-804.

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