UNCONVENTIONAL FORMS OF SYNAPTIC PLASTICITY
IN THE HIPPOCAMPUS AND THE STRIATUM

by

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Abstract

Synaptic transmission occurs as a result of either a spontaneous release of presynaptic vesicles or a batch release of presynaptic vesicles driven by action potentials. The physiological consequence of synaptic transmission driven by different patterns and frequencies of presynaptic stimulation has been extensively investigated. However, the physiological nature, mechanism as well as relevance of prolonged presynaptic stimulation have been poorly characterized. In this dissertation, I present three projects in which prolonged stimulation of synaptic transmission in different forms and different brain regions was studied for its effect on synaptic transmission, mechanisms and physiological relevance. In the first project, prolonged electrical stimulation (100 sec) at high frequency induced a deep synaptic depression in acute hippocampal slices, followed by a recovery of synaptic transmission after ~15 min. The deep synaptic depression was attributed to a complete depletion of presynaptic vesicle pools. In the second project, attempts were made to characterize the mechanism of nuclear activation of gene transcription induced by prolonged electrical stimulation (100 sec). Our results demonstrated that reduced inactivation of non-L-type calcium channels failed to provide calcium required for gene transcription, leaving the activation of gene transcription a selective function for L-type calcium channels. In the third project, we sought to study the physiological relevance of enhanced miniature events of inhibitory synapses induced by prolonged chemical stimulation. We showed that prolonged application (2 min) of nicotine to the striatal slice enhanced the frequency of miniature inhibitory currents that was accompanied with a reduction in the amplitude of evoked response. This reduction in the amplitude of evoked responses was ascribed to a compromised action potential invasion of presynaptic terminals possibly due to inactivation of sodium channels resulting from nicotine-induced depolarization. To summarize, prolonged stimulation of presynaptic vesicle release imposes significant influence upon neuron-to-neuron communication, with distinct mechanisms in different brain regions.

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<tr>
<td>ACSF</td>
<td>artificial cerebral spinal fluid</td>
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<tr>
<td>AP</td>
<td>action potential</td>
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<tr>
<td>APV</td>
<td>(2R)-amino-5-phosphonovaleric acid</td>
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<tr>
<td>AMPA</td>
<td>alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
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<tr>
<td>BG</td>
<td>basal ganglia</td>
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<tr>
<td>CNQX</td>
<td>6-cyano-7-nitroquinoxaline-2,3-dione</td>
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<tr>
<td>CREB</td>
<td>cAMP-Responsive Element Binding Protein</td>
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<tr>
<td>DIV</td>
<td>days in vitro</td>
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<tr>
<td>EEG</td>
<td>electroencephalography</td>
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<td>EPSC</td>
<td>excitatory postsynaptic current</td>
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<td>GFP</td>
<td>green fluorescence protein</td>
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<tr>
<td>GPe</td>
<td>globus pallidus external segment</td>
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<tr>
<td>GPi</td>
<td>globus pallidus internal segment</td>
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<tr>
<td>EBSS</td>
<td>Earle's Balanced Salt Solution</td>
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<tr>
<td>FS</td>
<td>fast-spiking</td>
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<tr>
<td>HEK</td>
<td>human-embryonic kidney</td>
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<tr>
<td>IPSC</td>
<td>inhibitory postsynaptic current</td>
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<td>nAChR</td>
<td>nicotinic acetylcholinergic receptor</td>
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<tr>
<td>MSN</td>
<td>medium spiny neuron</td>
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<tr>
<td>Nif</td>
<td>Nifedipine</td>
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<td>NMDA</td>
<td>N-methyl-D-aspartic acid</td>
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<tr>
<td>LTD</td>
<td>long-term depression</td>
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<tr>
<td>LTID</td>
<td>long-train induced depression</td>
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<td>LTP</td>
<td>long-term potentiation</td>
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<td>LTS</td>
<td>long train stimulation</td>
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<tr>
<td>RP</td>
<td>reserve pool</td>
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<tr>
<td>RRP</td>
<td>readily-releasable pool</td>
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<tr>
<td>SNpc</td>
<td>substantia nigra pars compacta</td>
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<tr>
<td>SNr</td>
<td>substantia nigra pars reticulate</td>
</tr>
<tr>
<td>STN</td>
<td>subthalamic nucleus</td>
</tr>
<tr>
<td>TAN</td>
<td>tonically active neuron</td>
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<tr>
<td>TBS</td>
<td>theta-burst stimulation</td>
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<td>TTX</td>
<td>tetrodotoxin</td>
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<td>VGCC</td>
<td>voltage-gated calcium channel</td>
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Dedication

This dissertation is dedicated to my dear parents, Zhonghui Liu and Hongjuan Ha, who have given me moral support throughout my study.
Co-Authorship Statement

1. Identification and design of research program: I have identified and designed all the work in the first project in the second chapter, under the guidance of my supervisor Dr. Tim Murphy. The identification and the design of the third chapter were performed by Dr. Tim Murphy for the initial design and by me for the technical arrangement. The project in the fourth chapter was designed initially by Dr. Otsu and Dr. Murphy and later by myself for technical arrangements for most of the experiments included in the paper during revision.

2. Performing the research: I performed majority of the research included in the second, the third chapter. For research included in the fourth chapter, I did all the work required during revision of the paper, modified and improved the existing data by adding more data points.

3. Data analysis: I performed all the data analyses included in the second, the third chapter. For analyses included in the fourth chapter, I did all the analyses required during revision of the paper, reanalyzed all the existing data pools.

4. Manuscript preparation: I performed all the writing involved in the first, second, third, the fifth chapter and the material included in the Appendix. For the fourth chapter, the co-first authors Dr. Otsu and Dr. Murphy wrote the first draft. I performed all the rewriting during revision and have added a few new sections in both results section and discussion section, with English errors corrected by Dr. Murphy.
Chapter I

Introduction

1.1 Overview

The synaptic plasticity, commonly thought as a cellular substrate for learning and memory in the CNS, takes on many forms in different brain regions. The long-term potentiation (LTP), perhaps the most thoroughly investigated in the hippocampus and the cortex, has been widely accepted as the building brick for memory formation (Castro et al. 1989; McNaughton et al. 1986). Depending upon the length and strength of conditioning protocols, LTP duration normally exhibits a great variation, from less than an hour to days, even months in some occasions (Abraham et al. 2002). Typically LTP is induced by a number of episodes of high frequency stimulation, separated with a short period of time, to ensure sufficient vesicle supply from presynaptic vesicle reservoir. In our study during my PhD period, the main research focus has been around the postsynaptic plasticity profile induced with prolonged stimulation at high frequency with no interruption, and the nuclear activation of gene transcription. Specifically we are interested in whether a possible depletion of presynaptic vesicle would affect the postsynaptic plasticity and what mechanisms might be involved.

The hippocampus and the striatum have been chosen as our experimental preparations. Since these two central regions share little structural similarity, different stimulation strategies have been designed respectively for these two regions. For experiments with the hippocampus, prolonged electrical field stimulation was delivered to the stratum radiatum where the Shaffer-Collateral-Commissural pathway is located. With a typical 100 sec high frequency stimulation we observed a deep depression of synaptic transmission that lasted about 20 min, followed by a normal LTP if the postsynaptic neuron was depolarized during the conditioning stimulation. With some supporting experiments, the observed synaptic depression was ascribed to depletion of presynaptic vesicles. For experiments with the striatum, we chose bath perfusion of nicotine as means of stimulating the inhibitory GABAergic transmission, mainly based upon the inhibitory nature of its main output for motor control and its non-laminar structure. The result showed that during the robust enhancement of GABAergic activity induced by prolonged application of nicotine (longer than 5 min), the evoked response was diminished significantly. Further analysis indicates that although prolonged high frequency activity tends to result in a depletion of
presynaptic vesicle pools in different brain regions which seems to serve distinct functions.
In particular, the vesicle depletion induced by prolonged electrical stimulation using theta
burst pattern in the hippocampus was manifested in a long synaptic depression that
perhaps provides a protection to the postsynaptic neuron from the potential harm with
further stimulation. However, in the striatum, possible depletion of presynaptic vesicles
induced by prolonged nicotine application, although likely incomplete, was suggested to
serve to dampen the evoked response. In the last experimental chapter, we were trying to
establish the possible involvement of L-type voltage-gated calcium channels (VGCCs) in
action potential (AP) train-induced activation of cAMP Responsive Element Binding
Protein (CREB). However our results did not lead to any conclusive suggestion.
1.2 What is Learning and Memory?

1.2.1 Learning

Based upon Eric Kandel’s quote: “Learning is the process by which we acquire knowledge about the world. (Kandel et al. 2000)” This definition, however, is a bit too general and doesn’t carry sufficient physiological sense in it. In my opinion, learning is the process in which we consolidate our memory in order to output a desired response. Although mentioned mostly with memory together, it differs from memory in that learning engages a complete cycle of a complex reflex while in contrast, memory only involves the information deposition in our brain. Based upon the research emphasis of my work, little will be discussed about learning.

1.2.2 Memory

Again based upon Kandel’s quote: “…memory is the process by which that knowledge of the world is encoded, stored, and later retrieved. (Kandel et al. 2000)” Memory can be categorized into two kinds, declarative or explicit and non-declarative or implicit memory or procedural memory. Declarative memory deals with things and facts that will be brought into consciousness to be used. It can be further sorted as episodic memory to describe events, for example, “We had a basketball game in our gym” and semantic memory to describe facts, for example, “paper is normally white”. Non-declarative or implicit memory deals with deposited information in our brain that does not engage our consciousness. This type of memory refers to those reflexive or perceptual skills after training and is recalled unconsciously. For example the memory of driving a vehicle is achieved by repeatedly learning how to drive. And we drive our car by retrieving the memory of how to drive without conscious recall. The implicit memory is further parceled as associative and non-associative memory. Non-associative memory is best represented and researched in invertebrate animals as Aplysia Californica’s gill withdrawal behavior, in which, a response is weakened after a session of repeated mild stimulation, habituation, and a response is strengthened after a single noxious stimulus, sensitization (Castellucci et al. 1989; Castellucci et al. 1978; Pinsker et al. 1973). In associative learning, two stimuli are associated with each other or an effective response depends upon the co-existence of two stimuli with certain sequence. Two types of associative learning have been studied: classical conditioning and operant
(instrumental) conditioning. In classical conditioning, as originally demonstrated by Russian physiologist Ivan Pavlov in 1890s, a trained dog salivates to the bell sound alone after the sound being associated with food. In operant conditioning, an animal learns to make an action that is associated with a reward or a noxious stimulus after repeated trials, with errors.
1.3 Structural Correlates of Memory

In general, no memory is processed in a single brain region. For explicit memory, memory traces are first acquired through one or more of the three polymodal association areas of the cerebral cortex, namely prefrontal cortex, limbic system and parieto-occipital-temporal cortex. Then, the information is transferred to parahippocampal and perirhinal cortices, entorhinal cortex, dentate gyrus, hippocampus, subiculum and back to entorhinal, parahippocampal and perirhinal cortex. Based upon lesion studies, there is greater memory loss in damage to perirhinal and entorhinal cortices than damage made to the hippocampus. While the left hippocampus has more to do with semantic memory, damage to the right hippocampus leads to more deficit in spatial memory (Kandel et al. 2000). For implicit memory, memory trace can be stored in different region depending upon how information is acquired. For example, amygdala is involved in ‘fear conditioning’ while operant conditioning involves striatum and cerebellum. The traditional notion has been that the hippocampus is only involved in formation of explicit memory. More recently it was found that implicit memory involves the hippocampus only when the presented information is new (Gooding et al. 2000).
1.4 Hippocampus

1.4.1 A historical patient that first revealed the function of the hippocampus

From clinical cases as well as experimental evidence collected over past years that involved lesion of different brain regions, we now know that different kinds of memory are stored in unique brain regions. Our knowledge about the hippocampus has been greatly benefited from the famous patient H.M., the process of whose treatment has made exciting advances in neuroscience and helped us to understand how memory is formed and retrieved. I would like to give a brief recollection of the story. On 23rd, August, 1953, the 27 year-old patient H.M. underwent surgery to remove both sides of medial temporal lobes in an attempt to treat his epileptic seizure. After surgery, seizure was much controlled. However, as a result of the surgery, he showed some shocking deficits of memory. There are two major dissociations of his memory deficit. One is the dissociation of old memory and newly formed memory. With intact memory for his life before 16 years old, H.M. has been experiencing a severe anterograde memory deficit that is still present nowadays. With 11 years retrograde memory loss, he can not form any new memory although the immediate memory is preserved in both verbal and non-verbal tasks. Another memory dissociation is about the declarative and non-declarative memory. H.M. exhibited an almost complete impairment of declarative memory while non-declarative memory was kept relatively intact. For example, H.M. was trained with a cognitive task. Although his task learning was relatively normal, he could not recall that he had ever been trained for the task (Milner 1968). Although the symptoms of patient H.M. suggest the important role of the hippocampus in learning and memory, especially in declarative memory formation and retention, no conclusion can be made specifically to the hippocampus since the surgery removed both the hippocampus and surrounding limbic cortex that is tightly associated to the hippocampal function.

In addition to declarative memory and implicit memory that is formed upon new presentation of test object, the hippocampus is specialized as well in spatial learning, as exhibited the most pronounced in water maze experiments (Jarrard 1978; Morris et al. 1982; Muller et al. 1987).
1.4.2 Inside the hippocampus

The hippocampus has a distinct structure that is readily identifiable at both the gross and histological levels. It is beautifully laminated; both the neuronal cell bodies and zones of the connectivity are arranged in orderly layers (Shepherd 2004). The hippocampus is an elongated structure located on the medial wall of the lateral ventricle, whose longitudinal axis forms a semicircle around the thalamus. The three dimensional position of rat hippocampal formation in the brain is shown in Figure 1.2. Due to its laminated organization, when the hippocampus is cut across its transverse axis (the septotemporal one), it is possible to identify a particular structure that is preserved in all slices taken with this orientation.

Figure 1.2: Position of hippocampal formation in the rat brain in which the cortical surface has been removed. The hippocampus is an elongated structure with the septotemporal axis running from the septal nuclei (S) to the temporal cortex (T). A slice cut perpendicular to
the long axis shows the well known three synaptic pathway (Adopted from (Amaral and Witter 1989), with permission from Elsevier. License number: 2014500156505).

The hippocampus proper and its neighboring cortical regions, the dentate gyrus (DG), subiculum and enthorinal cortex, are collectively termed “hippocampal formation”. As shown in Figure 1.2, the hippocampus proper is divided into stratum oriens, stratum pyramidale, stratum radiatum and stratum lacunosum-moleculare. In their classical works, Ramón y Cajal and Lorente de Nó grouped excitatory neurons in four regions called CA1-CA4. In general, CA4 is considered the part of the CA3 close to the dentate gyrus. The CA2 region represents the small portion between the CA3 and the CA1. This part is often ignored but it could have an important role in epilepsy because of the large amount of recurrent collaterals. All pyramidal neurons bear basal dendrites that arborize and form the stratum oriens and apical dendrites that are radially oriented in the stratum radiatum and lacunosum-moleculare. In the DG, granule cells represent the principal neurons, while the area between DG and the CA3 region is called the hilus (Figure 1.3).

**Figure 1.3:** Neuronal elements of the hippocampal formation. Labeled areas include the subiculum, part of the enthorinal cortex, the fornix, the dentate gyrus and the regions CA1 to CA4. The hippocampus proper is divided into stratum oriens, stratum pyramidale, stratum radiatum and stratum lacunosum-moleculare. (A drawing by Cajal, 1901. No permission required.)
1.4.3 Connectivity in hippocampus

The main inputs to the hippocampus come from the enthorinal cortex, the septum and the contralateral hippocampus, whereas a unique unidirectional progression of excitatory pathways links each region of the hippocampus, creating a sort of trisynaptic circuit (Figure 1.3). The perforant path, originating from the enthorinal cortex passes through the subicular complex and terminates mainly in the dentate gyrus, making synapses on granule cells. Then, the distinctive unmyelinated axons of the granule cells (mossy fibers) project to the hilus and to the stratum lucidum of the CA3 region. Here they make en passant synapses on CA3 pyramidal neurons showing the large, presynaptic varicosities typical of mossy fiber-CA3 contacts. These presynaptic expansions form a unique synaptic complex with equally intricate postsynaptic processes called thorny excrescences and may contain tens of releasing sites (Jonas et al. 1993). Information is therefore transferred, through Schaffer-Collaterals, from CA3 to CA1 pyramidal neurons, which send their axons to the subiculum and the deep layers of the enthorinal cortex. Then, signal is sent back to many of the same cortical areas. Thus, information entering the enthorinal cortex from a particular cortical area crosses the entire hippocampus and returns to the cortical area from which it was originated. The transformations that take place during this process are presumably essential for information storage (Shepherd 2004). Furthermore, commissural associative fibers provide synaptic contacts between CA3 pyramidal neurons and between the two hippocampi, via the fornix. In addition to connections to CA1 via Schaffer-Collaterals, CA3 pyramidal neurons also branch out collaterals back to themselves, which are the most numerous type of input to the CA3 pyramidal cells. These connections are responsible for generating epileptiform activity, characterized by spontaneous, synchronized and rhythmic firing in a large number of neurons (Miles and Wong 1986; Traub and Miles 1991). In contrast to the rather uniform population of excitatory neurons, local inhibitory interneurons, are widely distributed within the entire hippocampus. They have been differently classified according to their morphological, neurochemical and physiological characteristics, which include the intrinsic firing, network properties and activity dependent synaptic plasticity processes. They selectively innervate different domains of pyramidal cells, thus providing the main source of feedback and feed-forward inhibition (Freund and Buzsaki 1996; Miles et al. 1996). Due to their extensive dendritic and axonal arborization, GABAergic interneurons can phase the output of principal cells giving rise to a coherent oscillatory activity
(Klausberger et al. 2003; Klausberger et al. 2004; Somogyi and Klausberger 2005) thus exerting a powerful control on network excitability and information processing in the brain. Although some oscillations can be reproduced in vitro, they occur mainly in vivo during particular behavioral states of the animal (Buzsaki 2002; Buzsaki and Draguhn 2004). Oscillations have been implicated in encoding, consolidation and retrieval of information in the hippocampus (Freund and Buzsaki 1996).

1.4.4 Synaptic plasticity in hippocampus

Synaptic plasticity is a common physiological phenomenon at synapse level. The best way to define this important phenomenon is to bring up the pioneering work by the Canadian physiologist Donald Hebb (1949), with his famous quote:

“Let us assume that the persistence or repetition of a reverberatory activity (or "trace") tends to induce lasting cellular changes that add to its stability.…. When an axon of cell A is near enough to excite a cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A’s efficiency, as one of the cells firing B, is increased.”

This statement is normally summarized as ‘cells fire together, wire together’. This so-called Hebbian rule has formed the basis for studying mechanisms for learning and memory. From then on, massive efforts have been put into understanding how the strength of a connection between a postsynaptic cell and a presynaptic cell can be modified by a certain pattern of presynaptic input and how that would impact learning. Different patterns of synaptic activity have been known to generate distinct forms of synaptic plasticity. Almost all known forms of synaptic plasticity, among them long-term potentiation/depression, short-term potentiation/depression, facilitation, augmentation and post-tetanic potentiation (PTP), can be seen in hippocampus. Although synaptic plasticity is multifaceted, in this chapter LTP will be the main focus for discussion because LTP has been generally conceived as the substrate of memory, although debates in this regard remain a hot topic.

LTP was first discovered in 1973 in dentate gyrus synapses with inputs from perforant pathway (Bliss and Lomo 1973). At all the three major synapses in the trisynaptic circuitry of hippocampus, LTP has been observed although in different forms. Other than the hippocampus, LTP was also observed, in most of the central brain region. There are two distinct forms of LTP have been characterized in hippocampus. One is the widely expressed form, NMDA receptor-dependent LTP that is observed at perforant
pathway/dentate gyrus synapses and Shaffer-Collateral/CA1 (Collingridge et al. 1983). The other form is NMDA receptor-independent LTP that can be found at synapses between mossy fiber and CA3 pyramidal neurons (Harris and Cotman 1986). Pharmacology studies have indicated three distinct forms of glutamatergic receptors, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), N-methyl-D-aspartic acid (NMDA) and kainite receptor, all of which are involved in synaptic plasticity in various ways (Watkins and Evans 1981). Although less typical as a substrate for learning and memory, kainate receptor mediated-LTP represents an intriguing form of plasticity. Kainate receptors have been known for their presynaptic action on modulation of synaptic transmission. At low dose, they facilitate transmission while at high dose they depress transmission (Schmitz et al. 2000). However their role in LTP induction has not been clear for a long time because the mechanism has been obscured by the fact that mossy fiber LTP remains in the presence of broad spectrum glutamate receptor antagonists, such as kynurenic acid or 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (Castillo et al. 1994; Harris and Cotman 1986; Ito and Sugiyama 1991). At that time enough evidence have also been accumulated to conclude that at mossy fiber synapses there are even higher density of kainate receptors than NMDA receptors (Monaghan and Cotman 1982). Using a calcium/magnesium (5/4 Mm) concentration, it has been shown that mossy fiber LTP is independent of kainate receptors (Yeckel et al. 1999). A more careful investigation by Collingridge group using normal calcium/magnesium ratio in extracellular solution, they were able to demonstrate that presynaptic kainate receptors mediate mossy fiber LTP (Bortolotto et al. 1999). It turned out that the unblocked LTP in the presence of kainate receptor antagonist in the study by Yeckel et al was due to the compensatory effect by L-type calcium channels. To translate this phenomenon into realistic scenario, we think it is possible that significant amount of calcium influx via L-type calcium channels would require synaptic activity being elevated to a sufficient level that perhaps equates to a strong synaptic input such as a burst of AP volleys. Based mainly upon the proposal by Collingridge group (Bortolotto et al. 2005), I present the hypothesized model for mossy fiber transmission with slight modification:

Upon arrival of APs at the mossy fiber terminal, glutamate is release to postsynaptic AMPA receptors, as well as presynaptic kainate receptors by means of diffusion. Normal synaptic activity only activates postsynaptic AMPA receptors. Intermediate level of synaptic activity will induce more glutamate release that can not only activate postsynaptic AMPA receptors but also spread to presynaptic kainate receptors.
Strong synaptic activity can bring the presynaptic membrane potential to a sufficient level for activation of L-type calcium channels, possibly by both the depolarizing effect of sodium/calcium influx via kainate receptors and by the direct depolarization by AP volleys. Upon activation, kainate receptors may introduce sufficient calcium influx to induce regenerative calcium wave from intracellular calcium stores, resulting in long-term potentiation.

![Diagram of mossy fiber LTP](image)

**Figure 1.4:** A schematic model for the role of kainate receptors in mossy fiber LTP. (Adopted from (Bortolotto et al. 2005), with permission from Elsevier. License number: 2012160223821)

The most extensively studied form of LTP is from Shaffer-Collateral-Commissural pathway to CA1 pyramidal neurons. Four properties have been summarized from studies on these synapses. The first is the input-specificity. It states that LTP is restricted to the synapse that has been received LTP-inducing stimulation and does not spread to neighboring synapses (Andersen et al. 1977; Lynch et al. 1977). The second property is cooperativity. While weak tenani only activate insufficient number of afferent fibers, strong tenani can activate sufficient number of afferent fibers to generate a large postsynaptic depolarization to trigger induction of LTP (McNaughton et al. 1986). In cooperativity, the key parameter is the stimulation threshold for LTP generation. This threshold is a complex function of intensity and pattern of tenanic stimulation. The third property is associativity. It states that a weak tenani will become capable of triggering LTP if paired with a strong tenani from neighboring synapses (Levy and Steward 1979). The last property is persistence. LTP is long-lasting. In acute hippocampal slices, it has been firmly
established that LTP can last for hours. In free moving rats, LTP has been shown to last for months (Abraham et al. 2002).

To have a comprehensive understanding of LTP, one has to know how the LTP is induced, process of ‘induction’, what cellular elements express LTP (expression) and what cellular mechanisms support LTP expression (maintenance).

1.4.5 LTP induction

The process of LTP induction, which constitutes early events that initiate an increase in synaptic efficacy, is mechanistically distinct from the subsequent LTP expression and maintenance. During LTP induction, the delivery of high frequency stimulation to afferent fibers drives glutamate release into the synaptic cleft and depolarizes the postsynaptic membrane. A successful LTP induction relies upon two synaptic events that occur simultaneously, a presynaptic AP volley and a postsynaptic depolarization that is normally manifested in back-propagated APs. A key player in LTP induction is the NMDA receptor, which is uniquely suited to play a role in Hebbian synaptic plasticity, because maximal ion flow through this channel depends on the coincidence of two tightly related events. One is presynaptic activity, which releases glutamate to bind the receptor and the other is the postsynaptic depolarization, which is required to relieve blockade of the NMDA-R channel by extracellular magnesium (Bourne and Nicoll 1993). The other is postsynaptic depolarization. Upon arrival of tetanic stimulation, glutamate is released into synaptic cleft first to activate AMPA receptors which initiate permeation of cations such as sodium influx and potassium efflux, with different conductances to each ion, causing depolarization of postsynaptic membrane. Sufficient postsynaptic depolarization removes the voltage-dependent blockade of NMDA iono-pore by magnesium and thereby activates the NMDA receptor. Activation of NMDA receptors in this scenario relies upon the presynaptic activity being strong enough to simultaneously depolarize the postsynaptic membrane to threshold level to activation NMDA receptors. However, high frequency stimulation does not constitute the exclusive way of LTP induction. LTP can also be induced by low frequency stimulation of afferent fibers with paired postsynaptic depolarization by back-propagated APs or by artificially depolarizing current-injection (Sastry et al. 1986; Wigstrom et al. 1986). In both scenarios, there are two simultaneous events, presynaptic glutamate binding and postsynaptic depolarization that removes magnesium block. These two events open NMDA receptors and allow calcium ions enter the cell to initiate a transient calcium elevation in the cytoplasm. The
rapid elevation in cytosolic calcium triggers activation of some important intracellular enzymes that sustains early phase of LTP (E-LTP). The most important enzyme is CaMKII, which has been an important intrinsic property autophosphorylation that extends the effect of transient calcium elevation beyond the duration of stimulation (Fukunaga et al. 1993). NMDA receptor mediated calcium influx provides the calcium required for phosphorylation of CaMKII. The autophosphorylated form of CaMKII does not require calcium and becomes constitutively active for a certain period of time. A block of CaMKII function has been shown to impair the LTP almost completely (Giese et al. 1998; Otmakhov et al. 1997), suggesting that all the subsequent biochemical events that underlie NMDA receptor-dependent LTP are consequential to CaMKII activation. Another important enzyme for LTP is PKA. While CaMKII is critical for LTP induction, PKA plays a modulatory role in LTP induction. With normal stimulation intensity, PKA has been suggested to function as a gate for LTP induction (Blitzer et al. 1998). Upon heightened synaptic activity, PKA may become critical for late-phase LTP maintenance (Frey et al. 1993), which is dependent upon protein synthesis.

Figure 1.5: A scheme for NMDA receptor-dependent form of LTP induction (Original figure is included in (Collingridge 2003). Permission granted by Royal Society Editorial
1.4.6 LTP expression

The concept of LTP expression mainly refers to how and where the induced LTP is expressed. One of the major issues on this topic is where LTP is expressed on postsynaptic sites or on presynaptic sites. Evidence have accumulated that both postsynaptic and presynaptic mechanisms might be contributing to LTP expression. Major establishments that favor a postsynaptic mechanism of expression, include an increased AMPA receptor conductance resulting from GluR1 subunit phosphorylation by CaMKII (Benke et al. 1998; Derkach et al. 1999) and AMPA receptor insertion (Hayashi et al. 2000; Shi et al. 1999). Presynaptic expression mechanisms have also been proposed based upon the fact that LTP is associated with a decrease in synaptic failure with minimal stimulation paradigm (Isaac et al. 1996; Kullmann and Nicoll 1992; Malinow and Tsien 1990). However, the presynaptic view for LTP expression was later challenged with the discovery of silent synapses (Durand et al. 1996; Isaac et al. 1995). Silent synapse refers to a synapse with NMDA receptors, but not AMPA receptors. Delivery of AMPA receptors to a previously silent synapse converts the synaptic current from a failure to a success and can account for the proposed presynaptic expression mechanism for LTP. Although not conclusive, the most updated view for LTP expression is that LTP is primarily a result of modification of postsynaptic elements.

1.4.7 LTP maintenance

The concept of LTP maintenance has never been clearly defined, and mainly refers to the mechanisms that support the expression of LTP. For example, CaMKII can be regarded an element of LTP maintenance that supports both phosphorylation of AMPA receptors and the recruitment of new AMPA receptors from extrasynaptic sites to synaptic sites. For L-LTP, maintenance customarily refers to the processes related to protein synthesis, for example nuclear translocation of mitogen-activated protein (MAP) kinase and subsequent gene expression. In brief, the early phase of LTP is dependent on posttranslational modifications such as protein phosphorylation, later phases of LTP require new gene transcription and protein synthesis (Krug et al. 1984; Nguyen et al. 1994).
1.4.8 Long-term depression

Long-term depression (LTD) refers to the weakening of synaptic strength that lasts for hours to days. LTD can be induced with strong synaptic input such as occurs in the cerebellum (Ito and Kano 1982) or low frequency stimulation paired with moderate postsynaptic depolarization (Barrionuevo et al. 1980). LTD that occurs in different brain regions has distinct physiological implications. Cerebellar LTD has been hypothesized to be important for motor learning (Ito 1989). However, it is likely that other plasticity mechanisms play a role as well. Hippocampal LTD may be important for the clearing of old memory traces. Two types of LTD exist, homosynaptic and heterosynaptic LTD. Homosynaptic LTD refers to synaptic depression that occurs to a single synapse or a group of synapses that does not involve interactions between synapses, while heterosynaptic LTD is to the type of depression that involves interaction between synapses. One feature for homosynaptic LTD is that it is easily induced in a developing brain but requires special stimulation paradigms in adult brain (Kemp et al. 2000). In general, homosynaptic LTD is induced by a low frequency stimulation and is mediated by calcium influx via NMDA receptors (Dudek and Bear 1992; Mulkey and Malenka 1992). One important concern is how the same calcium signal mediates both induction of LTP and LTD. It turned out that LTP was triggered by a brief calcium transient with high magnitude while in contrast LTD was triggered by a persistent modest elevation of calcium in the cytoplasm via NMD receptors (Yang et al. 1999). With regard to receptor type at subunit level that mediates LTD, it was proposed that LTD-inducing stimuli trigger LTD via activation of NR2B-containing NMDA receptors (Liu et al. 2004). However, the result was not well reconciled with other studies. For example, overexpression of NR2B led to enhanced LTP in the hippocampus (Tang et al. 1999). It is possible that a more subtle change in NMDA receptor subunits may be involved in LTD maintenance, mechanisms of which remain a topic of investigation.

Heterosynaptic LTD was the first type of LTD that captured widespread attention before the homosynaptic LTD was reported (Lynch et al. 1977). In heterosynaptic LTD experiments in the hippocampus for instance, a tetanus induces homosynaptic LTP in the tetanized pathway while induces a synaptic depression in neighboring synapses in untetanized pathway, so termed heterosynaptic depression (Scanziani et al. 1996). One important clue for unveiling the mechanism of heterosynaptic LTD is the recent finding that extrasynaptic NR2B subunit of NMDA receptors mediate crosstalk between neighboring
synapses (Scimemi et al. 2004). However, this possibility has yet to be tested. Here we present our preliminary results on observation of heterosynaptic depression.

Figure 1.6: Our preliminary data showing the heterosynaptic LTD observed in an 18 day-old rat. Two stimulating electrodes were placed in Schaffer-Collateral pathway on either side of recording electrode that recorded the field potential of CA1 pyramidal neurons. Upon delivery of 3 bursts of 1sec 100Hz train with 30 sec interval, a persistent depression was observed at the unstimulated pathway. Group data are not presented due to the fact that LTP obtained in that set of experiments were mostly deteriorating.

1.4.9 Synaptic transmission and vesicle cycling

Synaptic transmission is the process underlying communication between neurons via a subcellular structure called synapse. The term ‘synapse’ was originally coined by Foster and Sherrington (Foster and Sherrington 1897; Tansey 1997). The word "synapse" comes from Greek: "syn" meaning "together" and "haptein" meaning "to clasp." Based upon the mechanism of transmission, there are two types of synapses, chemical synapse and electrical synapse. In this thesis, synapse exclusively refers to the chemical synapse. The process of the classic transmission can be broken up into four steps.

The first step is the storage of synaptic vesicles containing neural transmitters.
Upon arrival of an AP, the neuronal terminal possesses sufficient synaptic elements that renders a neuron capable of passing the information to the next neuron. The neurotransmitter is stored in small vesicle with a radius of 17-22 nm. These vesicles are scattered normally throughout the presynaptic terminal with a cluster of vesicles concentrated at the patch of membrane facing the synaptic cleft (Figure 1.7).

![Figure 1.7: Synaptic apparatus. The postsynaptic differentiation (arrowhead) is can be seen, as well as the uneven electron-dense material that fills a synaptic cleft about 20 nm wide. (X 115,000). From goldfish, Carassius auratus. (Adopted from J. Neurophysiol. Vol48, No3, 1982 with permission granted by APS permission administrator Penny Ripka)](image)

The second step is transmitter release. Some presynaptic vesicles are in a release-ready state and can be released immediately upon arrival of an AP. Upon arrival of an AP at a synaptic terminal, the AP depolarizes the presynaptic membrane and causes opening of VGCCs, predominantly N-type and P/Q-type channels at presynaptic sites (Dietrich et al. 2003). Then the elevation of intracellular calcium triggers the fusion of synaptic vesicles with the presynaptic membrane for exocytosis. Calcium triggers two modes of transmitter release. One is phasic release that is synchronous with calcium channel opening, lasting as short as 50 μs (Sabatini and Regehr 1996). Following the phasic release, there is a slower asynchronous release that lasts longer than one second, as a result of residual calcium (Atluri and Regehr 1998; Otsu et al. 2004). In general, the efficiency of exocytosis of synaptic vesicles is positively correlated with the level of intracellular calcium introduced by these calcium channels (Zucker 1999). In most terminals, only 10%–20% of APs trigger release. In the hippocampus in particular, the release probability averages 0.2~0.3 in
response to low frequency stimulation (Hessler et al. 1993), with considerable variability between synapses (Allen and Stevens 1994; Hanse and Gustafsson 2001).

The third step is transmitter binding onto postsynaptic receptors. The transmitter is first released into synaptic cleft where varying number of receptors reside on the postsynaptic side of the synapse depending upon the synapse type and brain region, or even specific region on one neuron.

To complete the transmission process, the released transmitter must undergo certain inactivation process after it finishes its mission such that the transmitter does not continually occupy the postsynaptic receptor. This is the fourth step, inactivation of neurotransmitter (for comprehensive review, see Sudhof 2004). Ways of terminating a synaptic transmission include simple diffusion, enzymatic degradation, transmitter reuptake by presynaptic transporter proteins and glial cell uptaking of the released transmitter.

Among these steps the neurotransmitter undergoes in its life cycle, the mechanism of how the synaptic vesicle is cycled has received tremendous attention and is thought to be under delicate modulation by many factors. It has been proposed that there are three vesicle cycling pathways including two fast pathways and a slow pathway (Sudhof 2004). One fast cycling pathway has a very short time course and is referred to as ‘kiss-and-stay’ mechanism, based upon observations pioneered a few decades ago. This mechanism suggests that after exocytosis, vesicles undergo endocytosis and refill rapidly, and that a subpopulation of vesicles associated with the active zone recycles locally (Barker et al. 1972). Another fast pathway has received more reputation due to its amusing name, ‘kiss-and-run’, which vividly describes a rapid endocytosis without the vesicular membrane being attached to the active zone (Ceccarelli et al. 1973). The slow cycling pathway is referred to as ‘endosomal recycling’ and describes the vesicle endocytosis induced by extensive stimulation such as to cause clathrin-dependent formation of parasynaptic cisternae and pits (Heuser and Reese 1973).
1.4.10 Synaptic vesicle pools and plasticity

Under low frequency stimulation, a postsynaptic neuron normally responds with a relatively steady success rate or amplitude depending upon the number of afferent fibers. In contrast, the release probability drops drastically and eventually reaches a low plateau, in response to a high frequency stimulation train. This low level of steady-state synaptic transmission has been thought to reflect the balance between continuing release driven by synaptic input and the replenishment of readily-releasable pool vesicles.

Traditionally vesicles are grouped into three pools. Vesicles seated at active zones are mostly primed (phosphorylated with ATPase) and are release ready, therefore called
‘readily-releasable pool (RRP)’. Vesicles from the RRP can be released and depleted in response to short-bursts of high frequency synaptic input (Otsu et al. 2004; Schneggenburger et al. 1999), a few milliseconds of depolarization (Mennerick and Matthews 1996), or a hypertonic shock in hippocampus buttons (Rosenmund and Stevens 1996). The other pool is the reserve pool (RP) that is somewhat away from active zone and represents a storage depot of synaptic vesicles. These vesicles constitute the majority (typically ~80–90%) of vesicles in most presynaptic terminals. They can be driven for release only in response to high intensity stimulation (Rizzoli and Betz 2005). I am listing a few of this kind since our project involves high intensity stimulation that might engage the involvement of release from the RP. In *drosophila* neuromuscular junction, mobilization of RP vesicles requires at least 5-10 Hz stimulation frequency in frog neuromuscular junction (Heuser and Reese 1973) or 30 Hz in *drosophila* neuromuscular junction (Delgado et al. 2000; Kuromi and Kidokoro 2000). Prolonged high potassium stimulation was reported to release vesicles from the RP in the Calyx of Held and possibly from the hippocampus as well (de Lange et al. 2003; Takei et al. 1996). The third vesicle pool, named ‘recycling pool’, refers to the vesicles participating in exo- and endocytosis during prolonged stimulation. This pool is thought to constitute 5-20% of the entire vesicle pool. It is comprised partly from the RRP and partly from the RP, as observed in hippocampal slices (Murthy and Stevens 1999).

**Figure 1.9:** Three vesicle pools. a) The classic three pool model. The RP makes up ~80–90% of the total pool, and the recycling pool is significantly smaller (~10–15%). The readily releasable pool (RRP) consists of a few vesicles (~1%) that seem to be docked and primed for release. b) Three kinetic components of release (indicating release of three vesicle pools) on depolarization of goldfish bipolar cells. The cell was stimulated in the presence of the styryl dye FM 1-43, and the increase in fluorescence gives a direct measure
of exocytosis. (extracted from (Rizzoli and Betz 2005) with permission from Nature Publishing Group, License Number: 2044640450590).

Release can be modulated by signals converging on synaptic terminals. Two presynaptic processes are principal targets subjected to regulation by multiple factors. One is the peak concentration of calcium that is induced by an AP. In this regard, any change that modifies the duration of APs, the open probability of VGCCs or the conductance of VGCCs is capable of changing the level of calcium transient induced by an AP (Qian and Saggau 1999). The other is the release probability in response to a given calcium concentration. The release probability in response to a given calcium concentration is highly variable and depends mainly upon two factors, the number of releasable vesicles and calcium-responsiveness of docked vesicles. In response to high frequency stimulation, a short-term depression is normally observed as in the case of paired-pulse depression with pair pulse interval shorter than 40 ms. This is generally attributed to depletion of immediately releasable vesicles. However, it is difficult to determine the weight of contribution from the number of available releasable vesicles and the calcium-responsiveness if the terminal is stimulated with a prolonged high frequency train.

One important objective in this thesis is to probe the synaptic plasticity with an emphasis upon presynaptic modulation of release probability, with prolonged high frequency stimulation. In this regard, we hypothesize that a thorough depletion of the entire presynaptic vesicle pool occurs in the midst of prolonged high frequency stimulation and, as a consequence, it may render the normal transmission depressed or silenced for a period of time. The recovery time of normal transmission is difficult to be predicted with some perplexing factors such as the possibility that vesicles from the large RP might be replenished prior to the refilling of the RRP vesicles. It is predicted that the postsynaptic plasticity induced by the lengthy stimulation will not be altered by the synaptic depression resulted from vesicle depletion.
1.5 CREB in Synaptic Plasticity

Our CNS normally undergoes constant modification of neuronal connectivity in response to environmental changes. Most of these changes in synaptic strength or addition of new neuronal connections requires new protein production. The central element for adaptive protein synthesis is mobilization of CREB protein. CREB is the abbreviation for cAMP-Responsive Element Binding Protein, which is a member of a family (CREB/ATF) of structurally related transcription factors that bind to promoter CRE sites. In mammals, at least three genes encode the CREB-like proteins, CREB, CREM (cAMP-Response Element Modulator) and ATF-1 (Activating Transcription Factor) (Foulkes et al. 1992; Hoeffler et al. 1988; Rehfuss et al. 1991). CREB has been implicated in a number of biological functions including memory formation, and all of these functions depend on its ability to act as a stimulus-induced transcription factor that can be activated by a variety of extracellular signals. With regards to memory formation, CREB is involved in formation of long-term memory in different species. In Aplysia, long-term facilitation of synaptic strength is blocked by injection of CRE oligonucleotides into the presynaptic nucleus, although short-term changes do not appear to share this sensitivity (Alberini et al. 1994; Dash et al. 1991; Kaang et al. 1993). In transgenic Drosophila, long-term memory disappears or is augmented upon induction of repressor or activator forms of CREB, while short-term memory appears to be intact (Tully et al. 1994; Yin et al. 1995; Yin et al. 1994). In mammals, it has been shown that mice with mutated CREB are deficient in both the L-LTP in the hippocampus and long-term memory, while short-term changes in synaptic strength and short-term memory are largely spared (Bourtchuladze et al. 1994). It seems that CREB plays a central and highly conserved role in the production of protein synthesis–dependent long-term changes in the CNS (Frank and Greenberg 1994).

In the CNS, CREB mediates both activity-dependent synaptic plasticity and trophic factor-dependent neuronal survival (Shaywitz and Greenberg 1999; Silva et al. 1998). Activation of CREB has been shown to be required for L-LTP but not E-LTP (Abel et al. 1997; Frey et al. 1993; Huang and Kandel 1994). However, more recent evidence have casted doubts on L-LTP dependence upon CREB activation as indicated in the observation that the conditional knockout mice with forebrain-specific deletion of CREB have completely normal L-LTP as well as E-LTP and performed normally in the Morris-water maze and fear conditioning (Balschun et al. 2003). In our opinion, evidence from knockout animals, although technically tempting, should be taken with care. In knockout animal, animals develop compensatory mechanisms to a varying degree.
Dependence of long-term consolidation of synaptic strength and memory upon CREB activation is mechanistically different in wild-type animal with CREB activation acutely inhibited from that in knockout animal in which a compensatory process can evolve gradually during development. Therefore, we think the normal L-LTP observed in conditional knockout mice with forebrain-specific deletion of CREB is not sufficient to reject the earlier results from wild type showing the critical dependence of L-LTP upon CREB activation (Frey et al. 1993; Huang and Kandel 1994).

The activation of CREB is critically dependent upon activation of PKA as indicated from studies with *Aplysia* (Fantozzi et al. 1994). However, in mammalian CNS, PKA inhibitors fail to block CREB phosphorylation driven by synaptic stimulation in cultured hippocampal neurons (Deisseroth et al. 1996). Instead, calcium is indicated to be critical in activation of CREB as reflected in the observation that calmodulin kinase (CaMK) inhibitor blocked synaptically driven CREB phosphorylation (Bito et al. 1996; Deisseroth et al. 1996), perhaps reflecting an evolutionary transition of CREB activation process. Ample evidence have proven that calcium is the key factor activating CREB in mammals. Calcium can enter the cytoplasm primarily via three routes to activate CREB, NMDA receptors, VGCCs (mainly L-type) and intracellular calcium stores. NMDA receptor-mediated calcium influx has been shown to play a critical role in the induction of LTP (Perkel et al. 1993) and also induce transcription of a number of immediate early genes (Bading et al. 1995; Cole et al. 1989). In hippocampal cultures, NMDA receptors have been shown to mediate calcium influx to induce CREB phosphorylation independent of VGCCs by APs (Deisseroth et al. 1996). In contrast, L-type VGCCs seem to play a more important role in induction of transcription of early genes. Blockade of L-type VGCCs only caused a moderate reduction in overall calcium influx, but led to a large reduction in transcription in immediate-early genes, suggesting that calcium influx via L-type VGCCs is tightly linked to gene expression (Murphy et al. 1991). The transcription of BDNF is also preferentially driven by calcium influx through L-VGCCs, whereas it is poorly induced by calcium entering through NMDA receptors (Ghosh et al. 1994). There have been considerable debates with regard to the route of calcium entry for gene expression. The majority of differences has arisen from observations made in either primary cultures or tissue slices. Related to CREB phosphorylation, activation of CREB has been shown to be independent of APs and activation of L-type VGCCs in primary hippocampal cultures (Deisseroth et al. 1996). In acute hippocampal slices on the other hand, CREB activation was demonstrated to be dependent upon APs and was blocked by L-type specific calcium
channel blockers (Dudek and Fields 2002). Results obtained from primary cultured neurons should be taken with extreme care and need to be confirmed with tissue slices or in vivo studies. Debates normally arise from a negative result in primary culture and a positive result from tissue slices. For another example, PKA blocked CREB-dependent transcription in hippocampal slices from the CRE-lacZ mice (Impey et al. 1996), but not in hippocampal cultures (Bito et al. 1996). The more intact neuronal circuitry preserved in tissue slices constitutes a rationale for people’s opinion leaning to observations obtained from tissue slices. Another calcium source is calcium store. Calcium release from calcium stores triggered by NMDA receptor-mediated calcium transients has been shown to relay the calcium signal into the nucleus which is critical for gene expression (Hardingham et al. 2001).

CREB can be phosphorylated at Ser133 position in response to a wide variety of stimuli. A number of signaling pathways have been proposed to result in the nuclear phosphorylation of CREB. Among these pathways are the calcium/calmodulin activated kinases and the Ras/MAPK pathway. Activation of VGCCs and NMDA receptors leads to the elevation of cytoplasmic and nuclear calcium levels and the activation of the CaMKs. Although CaMKs I, II, and IV all can phosphorylate Ser133, CaMK-IV, typically activated by membrane depolarization, seems to be the most relevant CaMK (Bito et al. 1996). CaMK-IV is localized to the nucleus, and the kinetics of its activation correlate in time with CREB phosphorylation and dephosphorylation at Ser133 (Bito et al. 1996). Another important signaling pathway is the Ras/MAPK pathway that is typically activated by nerve growth factor. One downstream substrate of the Ras/ERK pathway is a 90 kD ribosomal S-6 kinase-2 (RSK-2). Upon activation, both MAPK and RSK-2 translocate to the nucleus where they may phosphorylate CREB at Ser133 (Chen et al. 1992; Finkbeiner et al. 1997; Xing et al. 1996).

Phosphorylation of CREB is critical, but insufficient for activation of gene transcription since gene transcription can not be blocked by nuclear calcium chelation (Chawla et al. 1998). To complete the process required for gene transcription, another coactivator, CREB binding protein (CBP), is required. Phosphorylation of CREB recruits the CBP to bind to the CREB upon phosphorylation of CREB on its Ser133 position (Chrivia et al. 1993). CBP can interact with many transcription factors [36], providing a mechanism through which nuclear calcium could modulate the activity of a potentially large number of transcription factors and, consequently, the expression of many genes.

To summarize, a wide variety of external stimuli can mobilize distinct signaling
pathways that converge on activation of CREB. The Ras/MAPK/Rsk and CaMKIV pathways may work in concert in response to a particular stimulus with specific timing and magnitude, which can be translated into the type or amount of gene transcription driven by phosphorylated CREB. In addition, CBP interacts with a number of other transcription factors (for example, AP-1, NFκB, c-Fos, c-Jun) and transcription activator sites (for example Serum-Responsive Elements) (Arias et al. 1994; Kamei et al. 1996; Nordheim 1994). Therefore, transcriptional specificity may also be realized by competition for the availability of CBP among the signaling elements, culminating in a specific gene transcription.

As discussed above, L-type VGCCs are involved in induction of synaptic plasticity that particularly requires gene transcription. In our previous work, we demonstrated that the inactivation profile instead of the activation property constitutes the predominant factor shaping the functionality of the L-type VGCC whenever the channel is activated (Arias et al. 1994; Kamei et al. 1996; Nordheim 1994) (work generated during my Master study). With dramatically accelerated activation kinetics in experiments performed at physiological temperature, L-type VGCCs are capable of introducing significant calcium currents evoked by AP trains (Arias et al. 1994; Kamei et al. 1996; Nordheim 1994). As an extended attempt, we wanted to test whether AP train alone induces CREB phosphorylation independent of NMDA receptor activation, possibly via activation of L-type VGCCs. In this regard, the available evidence have been controversial (Deisseroth et al. 1996; Deisseroth et al. 1998). Then we will further address whether modification of inactivation profile of VGCCs will lead to a change in calcium influx sufficient to impose an influence on nuclear gene transcription as signaled with CREB activation. We hypothesized that a reduced inactivation of non-L-type VGCCs will lead to enhanced calcium influx from non-L-type VGCCs and subsequently increase the CREB phosphorylation to a detectable level. Unfortunately, our results turned out to be inconclusive. See chapter three for details.
1.6 Basal Ganglion

The basal ganglia (BG) are a collection of nuclei deep to the white matter of cerebral cortex. Nuclei comprising the BG include caudate, putamen, nucleus accumbens, globus pallidus internal segment (GPI), globus pallidus external segment (GPE), substantia nigra pars compacta (SNpc), substantia nigra pars reticulata (SNr), subthalamic nucleus. The putamen and the caudate are traditionally called the striatum, the name of which comes from its striated appearance created by radiating dense bundles of striato-pallido-nigral axons. The striatum is probably the most important structure in the BG, not only because it constitutes the primary input station but also due to the fact that many devastating diseases, such as the Huntington disease and some other related hyperkinetic movement disorders, affect this region. The globus pallidus internal segment (GPI) and the globus pallidus external segment (GPE) are collectively referred to as the palladium. After receiving input from cortices, the striatum sends signals to the GPI via a number of nuclei. Then the GPI relays signals to the thalamus, which sends signals back to cortices to complete the circuitry. Therefore, the GPI is thought to be the primary output station before signals are sent out of the BG. The pallidum is the site of therapeutic lesion (pallidotomgy) and deep-brain stimulation procedures used to relieve Parkinson’s disease. The subthalamic nucleus is a key structure controlling pallidal function, and is an increasingly favored site for deep-brain stimulation in the treatment of Parkinson’s disease. The substantia nigra is comprised of a compact (SNpc) and a diffuse clustering (SNr) of neurons. Both clusters lie dorsal to the cerebral peduncle in the ventral midbrain. The SNpc contains large dopaminergic cells that provide the dopaminergic input to the striatum also referred to as the nigro-striatal dopaminergic system.
Figure 1.10: Location of the basal ganglia in human brain. The putamen and caudate nucleus are located beneath the cerebral cortex in the midbrain. The globus pallidus is positioned on the inner surface of the putamen adjacent to the thalamus. (Figure adopted from (Calder et al. 2001). Permission obtained from Nature Publishing Group. License Number: 2044631429048)

In general, the function of the BG is described as a ‘brake’ in motor control. The ultimate effect on the thalamus is inhibitory after signals being relayed by a number of nuclei in the BG although there are many different neurotransmitters used within the BG including both excitatory and inhibitory (principally ACh, GABA, glutamate and dopamine). For example, to sit still, a person has to put brakes on all muscles except those used for maintaining an upright position. To initiate a movement, the person has to release the brake on muscles used for the voluntary movement while put brakes on unrelated muscles.

1.6.1 Direct pathway and indirect pathway

There are two major pathways through the basal ganglia. The direct pathway begins with projections from the cortex to the striatum. One population of striatal neurons projects directly to the GPi. The GPi projects to the thalamus which then projects back to the cortex. The excitatory connections from the cortex to the striatum use glutamate while
the striatum sends inhibitory signal to the GPi using GABA as the neurotransmitter. The connections from the thalamus back to the cortex are excitatory with glutamate as the neurotransmitter. The cortex excites the striatum which then inhibits the GPi through the direct pathway. The GPi is normally tonically active and inhibitory to the thalamus. When the GPi is inhibited, the thalamus is relieved from inhibition (this is called disinhibition, or releasing the ‘brake’) and excites the cortex, thereby reinforcing the desired voluntary movement.

The indirect pathway involves two additional BG nuclei, the GPe and the subthalamic nucleus (STN). As in the direct pathway, the circuitry starts with the cortex sending an excitatory signal to the striatum. Instead of sending the signal directly to GPi, the striatum first relays inhibitory signals to the GPe, which in turn inhibits the STN. The STN then sends excitatory inputs to the GPi, which inhibits the thalamus. The thalamus then sends the excitatory signal back to the cortex as in the direct pathway. Therefore, the final effect of activation of the indirect pathway is inhibitory to movement.

Among the two pathways, the direct pathway is known for its overall excitatory output to the thalamus then to the cortex, i.e. releasing the brake to the desired movement. The indirect pathway sends the inhibitory output to the cortex. What is missing here is the element that controls the balance between the two pathways. It is dopamine, released from the SNpc.

As many scientific findings were inspired by the urge of seeking therapeutic solutions for diseases, the discovery of dopamine function in the BG was also motivated by the search of treatment for the Parkinson’s disease. The first report on dopamine’s function in the BG was from an early study showing a dopamine deficiency in the Parkinson’s disease (Ehringer and Hornykiewicz 1960). What was followed immediately after the discovery of dopamine function in the Parkinson’s disease was the effective administration of levodopa for treatment of the disease, by the same group led by Professor Ehringer (Birkmayer and Hornykiewicz 1961). What is worth noting here is that such a professor who has changed the way of how the pharmacology can be practiced in a revolutionary manner, has been overlooked by the Nobel Foundation awarding committee from the year 2000 nominee list. Following the announcement of the prize winner, a letter was sent to the award committee, signed by 250 neuroscientists expressing their dissatisfaction (Helmuth 2001).

Dopamine is released in the striatum from neuronal terminals coming from the SNpc. The overall effect of dopamine on motor control is to release the ‘brake’, i.e.
sending an excitatory output to thalamus. The receiving neuron in the striatum is a type of GABAergic neuron called medium spiny neurons based upon their appearance, abbreviated as ‘MSN’ normally. In the direct pathway, the net effect of dopamine is excitatory while it is inhibitory in the indirect pathway. The fact that overall effect of dopamine for motor function is excitatory raises the question how the same group of MSNs differentially responds to the same neurotransmitter (dopamine) with either excitatory or inhibitory consequence. The answer lies in the discovery of two distinct types of dopamine receptors, D1 and D2 receptors. Both the D1 and D2 dopamine receptors are G-protein coupled receptors. When dopamine is bound to the D1 receptor, a second messenger-signaling cascade is initiated, resulting in depolarization of the neuron. When dopamine is bound to D2 receptors, in contrast, a second messenger cascade resulting in neuronal hyperpolarization occurs. Thus, the action of D1 receptors is to enhance cortical-striatal excitatory connection whereas the action of D2 receptors is to reduce corticostriatal inhibitory connection (Sealfon and Olanow 2000). There are evidence showing D1 receptors are on a special group of neurons in the striatum (Aosaki et al. 1998) while contrasting results demonstrated that two receptors are well colocalized on the same group of MSNs arguing that receptor type constitutes the element differentiating the effect of dopamine (Aizman et al. 2000). The two aspects of dopamine action work together to send a net excitation to the striatum, perhaps in a synergistic manner, releasing the ‘brake’ on muscles for a particular voluntary movement. Therefore, the direct pathway with its excitatory influence and the indirect pathway with its inhibitory influence is balanced under a constant modulation by dopaminergic inputs from the SNpc. A challenging question is how the modulation of striatal function is executed by the dopaminergic neurons in the SNpc. What we do know to date is that the SNpc receives reciprocal input from the striatum, as a primary source of innervation (Gerfen 1984; Holstein et al. 1986), although a small population of SNpc neurons receives inputs from subthalamus nucleus and the prefrontal cortex as well (Carr and Sesack 2000; Smith and Grace 1992). How these neurons in the SNpc fine-tune signals from the above structures remains to be investigated. Resolution of this question will no doubt provide us with some better ways of treating the Parkinson’s disease than the overall administration of levodopa.
Figure 1.11: Basal ganglia circuitry in normal conditions. In normal brain, parallel neuronal networks of the striatum connect and integrate functions between basal ganglia nuclei, various regions of the cerebral cortex and the thalamus. In the motor circuit, the motor cortex project to the putamen, where they synapse through excitatory glutamatergic neurons onto the MSNs. These striatal neurons are organized into two pathways: the ‘direct’ and the ‘indirect’ pathway. The direct pathway connects the striatum to the GPi and the SNr. The GPi and SNr are the output nuclei of the basal ganglia (GPi/SNr) and project to the brainstem and the thalamus and from the latter to the cortex. The influence of the GPi and SNr on the thalamus is inhibitory, whereas the thalamic projection to the cortex is excitatory. The indirect pathway also connects the striatum to the output nuclei of the basal ganglia but these fibers first pass through synaptic connections in the GPe and then the STN. Output from the STN to the GPi/SNr is excitatory. Excitatory projections are shown in blue; inhibitory connections are shown in black. PPN stands for pedunculopontine nucleus (adopted from Figure 2. in article “Modern therapeutic approaches in Parkinson’s disease” published in Expert Reviews in Molecular Medicine, Vol. 5: pp. 1-20, 2003, by Simon J.G. Lewis, Maeve A. Caldwell and Roger A. Barker. Permission granted by.
1.6.2 Balance between Cholinergic and dopaminergic system in the striatum

The motor function is constantly tuned by a balanced control of facilitation and inhibition, executed by the direct pathway and the indirect pathway. Under basal conditions, tonic inhibitory signals are sent from the striatum to thalamocortical neurons with a high resting activity (~90Hz), thus keeping the cortical motor neurons under tonic inhibition (Grillner et al. 2005; Hikosaka et al. 2000). There has been controversy on how a movement is initiated. The MSNs have a low-activity profile during resting conditions with a hyperpolarized membrane potential due to a highly expressed inward-rectifier potassium channel (Kir). These Kir channels tend to keep the MSNs in a stabilized, hyperpolarized ‘down state’ unless they receive a disinhibitory input (Surmeier 2004; Wilson and Kawaguchi 1996). When the Kir channel is deactivated, a plateau of depolarization is induced, called ‘up-state’. Once received inputs from the striatum, the SNpc sends the dopaminergic input back to MSNs via D1 receptors, facilitating transition of MSNs from ‘down state’ into ‘up state’ (Blackwell et al. 2003; Surmeier 2004). Activation of D1 receptors is implicated in depolarization of striatal neurons (excitatory) while D2 receptor activation tends to hyperpolarize the innervated neurons (inhibitory) (Sealfon and Olanow 2000). With regard to how these two distinct types of receptors balance between the excitatory direct pathway and the inhibitory indirect pathway, evidence have shown a differential distribution of two receptor types. Those striatal neurons projecting directly to the output nuclei of the BG have D1 receptors whereas those striatal neurons projecting indirectly, through other BG nuclei to output nuclei, have D2 receptors on their dendrites (Gerfen et al. 1990). It seems to fit well into the two pathway hypothesis. However, it cannot, by far, complete the interpretation for striatal control of motor function. Other evidence have suggested a colocalization of D1 and D2 receptors on the same group of striatal neurons (Aizman et al. 2000). Importantly, cholinergic neurons also play a pivotal role in striatal motor control. Traditionally it has been thought that motor function is under constant control from counteracting cholinergic system and dopaminergic system. There are two major cholinergic subsystems within the telencephalon. One is the projection subsystem that is composed of various basal forebrain nuclei. Those cholinergic neurons make broad projections throughout the cortex and the hippocampus. The second major cholinergic subsystem of the telencephalon is in the striatum. These cholinergic neurons
in the striatum belong to one of three major striatal interneurons. Details are not provided in this dissertation for the other two types of interneuron groups containing GABA, parvalbumin, somatostatin, neuropeptide Y, and NADPH diaphorase as potential transmitters (Gerfen and Wilson 1996; Kemp and Powell 1971). The cholinergic interneurons are large aspiny in appearance (somatic diameter in excess of 40 μm). In rats, the ratio of MSNs to large cells (mostly cholinergic interneurons) is about 100 to 1 or 2 (Oorschot 1996), which would make the cholinergic cells about 10–20% of all striatal interneurons (Graveland and DiFiglia 1985). In vivo intracellular recordings show that the cholinergic interneurons typically fire slowly and regularly, with APs of long duration and lengthy and slow spike afterhyperpolarizations (Wilson et al. 1990). This characteristic firing pattern is why these neurons are commonly known as TANs (tonically active neurons). In terms of motor control, these TANs have been implicated in responding to ‘GO’ signal for ‘brake’ release (Kimura et al. 2003). Although they fire mostly in this tonic mode, in vivo and in vitro recordings have shown that they exhibited a variety of firing patterns in response to different stimuli (Bennett and Wilson 1999), perhaps commanded by specific cortical inputs in order to release the ‘brake’. Although the overall effect of the cholinergic system and the dopaminergic system appears to be opposite, full understanding of their interaction has proven to be difficult. Activation of the corticostratial pathway leads to activation of both dopaminergic and cholinergic system in the striatum (Partridge et al. 2002). High frequency stimulation of the corticostratial terminal resulted in a dopamine-dependent LTD in the striatal neurons. The large aspiny cholinergic neurons in this study appeared to facilitate the striatal LTD by promoting the dopamine release in a presynaptic fashion (Partridge et al. 2002).

In this study, we were particularly interested in the role of nAChRs since evidence from multiple perspectives have proven its effects in improving learning and memory (Jones et al. 1999; Levin et al. 2006; Levin and Simon 1998; Newhouse et al. 1997; Picciotto et al. 1995; Potter and Newhouse 2004). Nicotine has been shown to enhance the fast synaptic transmission in the habenula of chick and the hippocampus of rat via a presynaptic mechanism (Gray et al. 1996; McGehee et al. 1995). In the hippocampus, both inhibition and disinhibition have been observed with exogenously applied ACh with majority of them exhibiting inhibitory effect by a presynaptic mechanism via the GABAergic interneurons (Ji and Dani 2000). We decided to further characterize the role of nAChRs in shaping synaptic plasticity in the striatum where the importance of nAChRs has been implicated in degenerative neurological diseases as the Parkinson’s disease.
The specific aim was to investigate how activation of nAChRs will affect GABAergic minis and consequently influence AP signaling across synapses as reflected by eEPSCs. Our result indicated a greatly enhanced GABAergic activity by exogenously applied nicotine and diminished eEPSC amplitude, perhaps suggesting a mode switch mediated by miniature activity-mediated GABAergic tone. (Hebb 1949)
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Chapter II

Prolonged Theta-Burst Stimulation Induced 20 min of Synaptic Depression at Hippocampal CA3-CA1 Synapses

A version of this chapter will be submitted for publication. Zhi Liu, Majid H. Mohajerani, Austen J Milnerwood, Yutian Wang and Timothy H. Murphy. prolonged theta-burst stimulation induced 20 min of synaptic depression at hippocampal CA3-CA1 synapses.
2.1 Summary

Various stimulation paradigms have been administered for LTP induction. In an attempt to deplete the entire presynaptic vesicle pool we have examined the effect of prolonged theta burst stimulation (TBS) on direction and extent of synaptic plasticity in hippocampal slices. By applying a 100-sec long TBS (holding potential: -70mV) to the Schaffer-collateral pathway at minimal intensity, postsynaptic CA1 pyramidal neuron exhibited a surprisingly long depression of excitatory postsynaptic currents (EPSCs) that lasted up to 27 min (19±1.4 min, N=11). Recording of back-propagated APs from CA3 pyramidal neurons revealed faithful AP firing after the 100 sec TBS, suggesting that observed synaptic depression was not an artifact resulting from conduction failure, but instead is a form of synaptic plasticity not reported previously. To determine the locus of the depression, dual EPSCs mediated by AMPA and NMDA receptors were recorded. Both AMPA and NMDA receptor-mediated EPSCs displayed a similar pattern of synaptic depression in parallel after the 100 sec TBS, supporting a presynaptic nature of the observed depression. When the holding potential was set to 0 mV during the TBS, LTP (net potentiation: 126.7±22.1%, N=7, P<0.001) was observed following the presynaptic depression with a noticeably shorter duration (5.7±1.8min, n=7, P=0.007) compared to that in control group (-70mV holding potential at conditioning), suggesting the postsynaptic function is not altered by the depression. Shorter theta-burst stimulation (5sec, 10sec) diminished the synaptic depression. Based upon previous studies that suggested distinct stimulating frequencies drive separate vesicle pools (Kuromi and Kidokoro, 2000), we applied a short burst of 20Hz train in the middle of the synaptic depression, 10min after the conditioning. The short burst of 20Hz train partially resumed synaptic transmission during the depression period, suggesting a partial replenishment of vesicles that are not readily releasable during the synaptic depression. Our results suggest that prolonged high frequency stimulation depresses the normal synaptic transmission for long time perhaps without alteration of postsynaptic functions. Before paper submission, a few more experiments will be performed to strengthen the finding. (Project supported by a grant from CIHR)
2.2 Introduction

Activity-dependent change of synaptic strength has been generally viewed as a plausible neural basis for learning and memory (Hebb 1949; Kandel and Schwartz 1982). Synaptic plasticity has been sorted into different categories based upon the induction paradigms (Martin et al. 2000). Among them, long-term potentiation (LTP) has received the most extensive attention as a possible neural substrate for memory formation. Different stimulating paradigms have been applied effectively for LTP induction (Hernandez et al. 2005; Yun et al. 2002). The most extensively investigated form of LTP is that exhibited by synapses between CA3 and CA1 pyramidal neurons in the hippocampus. LTP at these synapses is typically induced by episodes of high frequency (100 Hz) stimulation (HFS) or theta burst stimulation (TBS) (Bashir et al. 1991; Buzsaki 2002; Capocchi et al. 1992; Yun et al. 2002). Increasing numbers of TBS episodes produce a U-shaped LTP profile, but the question remains open as whether a single train of TBS with varying length would result in a similar outcome (Abraham and Huggett 1997). All the previous investigations on LTP have one practice in common, i.e. using conditioning episodes with sufficient intervals to allow replenishment of the presynaptic vesicles. We sought to investigate the plasticity profile when a single high frequency stimulation is delivered without interruption to the CA3-CA1 synapses. What makes this scheme particularly tempting is the possible hindrance of LTP induction by the potential depletion of entire presynaptic vesicles instead of depletion of only the RRP with a prolonged stimulation. It is known that a short-term depression is typically seen in synaptic responses during a short high frequency train due to depletion of vesicles from the RRP (Zucker and Regehr 2002). Synaptic transmission recovers quickly in a scale of seconds, or even milliseconds after a short burst of high frequency train or even stronger stimulant like an episode of hypertonic sucrose application (Betz 1970; Del Castillo and Katz 1954; Otsu et al. 2004; Thomson et al. 1993; Varela et al. 1997). The recovery is hypothetically realized by vesicle recycling and recruitment of vesicles from the RP. The recovery after prolonged high frequency stimulation remains an untouched issue for hippocampal synapses. Early studies have shown a reduced postsynaptic current that sustained for minutes after a prolonged high frequency train, suggesting a reduced presynaptic release due to depletion. However these studies were performed mostly on synapses with high fidelity requirement for transmission, e.g. neuromuscular junction, auditory synapses in the Calyx of Held or Mauthner fiber-giant fiber synapse of the hatchefish (de Lange et al. 2003; Glavinovic 1995; Heuser and Reese 1973; Highstein and Bennett 1975) where
presynaptic vesicles are capable of sustaining high frequency stimulation with high fidelity with little synaptic fatigue. Therefore it is of particular interest to see the synaptic transmission profile after the prolonged high frequency stimulation at a low fidelity synapse like the CA3-CA1 synapse. We hypothesized that a prolonged TBS would deplete the entire presynaptic vesicle reservoir that may manifest in a period of depression followed by a possible LTP. To our surprise, the depression after the prolonged TBS conditioning was extraordinarily long, lasting up to almost 30min followed by LTP with the 100 sec long TBS train if the membrane potential was held at a depolarized level during the conditioning. Combining our additional supporting data, the results suggest that prolonged high frequency conditioning abolishes post-tetanic potentiation (PTP) and prompts the development of a long presynaptic depression, which may represent a novel form of plasticity.
2.3 Materials and Methods

2.3.1 Slice preparation

Male or female Wistar rats (postnatal day 21-28) were anaesthetized with halothane and decapitated. All animals used in this project were cared for in accordance with regulations of the Canadian Council on Animal Care. The brain block containing the hippocampus was placed into chilled, modified artificial cerebrospinal fluid (modified ACSF) with composition (in mM) of 124.0 NaCl, 27.0 NaHCO₃, 2.5 KCl, 1.0 CaCl₂, 10.0 MgSO₄, and 11.0 D-glucose, equilibrated with 95% O₂-5% CO₂ (pH 7.45, 305 mOsm). Transverse slices of the hippocampus and its surrounding cortex (250-300 μm thickness) were prepared with a vibrotome (LEICA VT1000S) and incubated in an incubation chamber at room temperature for at least 1h in normal ACSF prior to being transferred to a recording chamber perfused with normal ACSF containing (in mM): 124 NaCl, 2.5 KCl, 2.5 CaCl₂, 1.3 MgCl₂, 1.25 NaH₂PO₄, 25.0 NaHCO₃, 11 glucose (pH 7.40 with 95 % O₂ / 5 % CO₂, 300 mOsm). CA3 region was separated from CA1 region with a small cut to prevent re-current epileptic activity.

2.3.2 Electrophysiology

Hippocampal CA1 pyramidal neurons were recorded with method of either blind patch or visualized path using a water immersion objective lens (Olympus 5X, 60X), in a submersion recording chamber that was constantly perfused with picrotoxin-containing ACSF (picrotoxin, 100uM) at 2ml/min perfusion speed. Data acquired with blind and visualized patch are pooled. A whole-cell patch electrode (~4 MΩ) was used to record synaptic responses from these neurons in a voltage- or current-clamp mode with an Axopatch 200B. Series resistance was compensated 60-70%. For voltage-clamp recording, the routinely used the internal solution contained (in mM): 130 Cs-methane sulfonate, 10 HEPES, 2 CsCl, 0.2 EGTA, 4 MgATP, 0.3 Na₂GTP and 5 QX314-Cl. The pH of the two internal solutions was adjusted to 7.25 by CsOH and the osmolarity was adjusted to 290 mOsm. The liquid junction potential was not corrected. When responses were monitored with current-clamp mode, the composition of internal solution was changed as follows (mM): 129.4 K-gluconate, 10 HEPES, 11.1 KCl, 0.02 EGTA, 4 NaCl, 3 MgATP, 0.3 Na₂GTP (pH adjusted to 7.25 using KOH). Recordings were all performed at room temperature. A mono-polar glass electrode containing normal ACSF was placed in
the Schaffer-Collateral path 50-100 µm below and 50-100 µm lateral to the recorded neuron.

![Schematic demonstration of recording and stimulation in the hippocampus.](image)

**Figure 2.1:** Schematic demonstration of recording and stimulation in the hippocampus.

Stimulation paradigms for both baseline and conditioning recording were programmed in Clampex software and were fed to a MASTER-8 stimulator where the stimulation pulse width was set up 100 µs. The pulse was finally forwarded to a stimulus isolator (World Precision Instrument, A385) where the stimulation intensity was adjusted. In a typical experiment, a 10 min baseline was recorded with the test stimulus applied every 20 sec. This test frequency was used for both baseline and post-conditioning recording. The theta frequency was used as conditioning paradigm, with 5 Hz bursting frequency and 4 pulsed in each burst with 50 Hz intra-burst frequency. Throughout the project, the stimulation intensity was standardized to an arbitrarily defined ‘minimal stimulation’ that resulted in a mixture of success and failure of excitatory post-synaptic current (EPSC), with an approximate failure rate of 30-50%. Signals were digitized at 5 kHz and filtered at 2 kHz (low-pass Bessel filter). Drugs used were all obtained from Sigma-Aldrich unless otherwise stated.
2.3.3 Statistical analysis:

Unpaired student t-test was performed for data sets with two groups of points with assumed equal variance, using ‘ORIGIN’ software. For data with three or more related groups we used one-way ANOVA with Bonferroni post-hoc testing in ‘PRISM’ software. Data are expressed as mean±S.E..
2.4 Results

2.4.1 Short-term depression induced by prolonged theta burst stimulation

The primary aim in this study is to examine the plasticity profile with an unconventionally long TBS that may lead to depletion of presynaptic vesicles in hippocampal slice preparation. We first looked into the response profile during the long conditioning with potential held at -70mV at which level sufficient driving force drives cations going through AMPA receptors at ligand binding and keeps NMDA receptors blocked with magnesium occupying in the channel pore. During a ‘minimal stimulation’, each theta burst consistently induced at least one evoked EPSC for only less than typically 2 sec followed by sparsely evoked responses. In contrast, the ‘normal stimulation’ with 100% above the threshold intensity induced much longer consistent firing of evoked EPSC (~20 sec) and stabilized at higher frequency after the termination of the consistent firing. Once the sealed cell was ruptured into whole cell mode, the intensity of stimulation was adjusted as quickly as possible such that a 30-50% of EPSC failure was obtained. The entire period from cell rupture to TBS conditioning was controlled within 15 min to minimize washout of cellular elements that might be necessary for LTP induction. For all the cells recorded, the pre-TBS baseline was at least 10 min long with only the last 10 min baseline was used for analysis. Immediately after the 10 min baseline a 100 sec long TBS was delivered to the neuron followed by at least 40 min long post-conditioning recording. To test the plasticity profile with 100 sec TBS, the pre-TBS baseline and post-TBS recording had a holding potential of -70 mV while the membrane was depolarized to 0 mV during the entire course of TBS conditioning. Since we were using minimal stimulation for invoking activation of only a small number of synapses, the amplitude variation in responding to either individual stimulus or train stimuli is consistently large, probably reflecting a high degree of heterogeneity of functional state for recorded pyramidal neurons. To our surprise, the stimulation resulted in a peculiar EPSC response profile with normal LTP preceded by a period of depression. With no exception, no PTP was observed. Some of neurons showed a deep depression, sometimes a synaptic silence for up to 10 min immediately after the delivery of the 100 sec TBS as shown in the example plot in Figure 2.2. A total of 7 cells were tested for LTP induction and plotted on the right in Figure 2.3. The potentiation was evaluated by comparing the averaged EPSC peak during the 10 min baseline with EPSC averages during the 10 min from 30 to 40 min after conditioning.
as percentage increase. In this thesis, ‘conditioned synaptic strength’ will be used for describing the percentage increase of the above-mentioned EPSC average over at least 10 min recorded 30-50 min after conditioning. As shown in Figure 3B, 100 sec TBS at 0 mV depolarization level induced a relatively stable potentiation about 10 min following the TBS conditioning (Percentage increase=126.7±22.1%, N=7, P<0.001 when compared with baseline averaged peak). LTP induction was also attempted with 10 sec TBS conditioning. Details of experiment rationale and analysis will be provided in later sections. To evaluate the time course of the depression we measured the time course required for EPSC amplitude to recover to 30% of the conditioned synaptic strength. This period is termed ‘depression period’ in this thesis. The depression period for this LTP group is 5.7±1.8 min (N=7).

**Figure 2.2:** The standard stimulation paradigm and resulted EPSC traces. Upper panel, 100 sec long theta burst stimulation paradigm. Middle panel, EPSC traces showing the first and the last 5 sec of EPSC traces during the theta burst stimulation with the stimulation intensity about 100% above the threshold stimulation, which is termed ‘strong stimulation’. Lower panel, the first and the last 5 sec of EPSC traces during the theta burst stimulation with the stimulation intensity set so as to induce a mixture of successes and failures of evoked EPSCs during baseline recording with the failure rate of approximately 30-50%. For both middle and lower panel, the holding potential was -70 mV.
Figure 2.3: Synaptic plasticity induced by TBS at minimal stimulation intensity. A and B show example plots of synaptic plasticity induced by 10 sec (A) and 100 sec (B) of TBS conditioning during which the membrane potential was held at 0 mV. Note the apparent lack of initial depression immediately after the 10 sec TBS conditioning in A. C, the group data showing effect of both 10 sec and 100 sec long TBS conditioning on evoked EPSC amplitude. Each grouped point was first normalized to the average of eEPSCs during baseline recording and then, for a visual clarity regrouped, by averaging normalized amplitude value of six temporally adjacent eEPSCs. D, the same set of data with different plot method, with the error bar spared to present a better visual clarity.

The difference of the response profile between results obtained with 10 sec versus 100 sec TBS conditioning suggested that the observed depression immediately after the 100 sec TBS conditioning was a result of the presynaptic vesicle depletion. To test this possibility, similar experiments were repeated with the membrane potential commanded at -70 mV during the period of TBS conditioning as well as during synaptic strength testing before and after the conditioning in an attempt to observe synaptic change without the involvement of NMDA receptor activation. In reality, it is impossible to hold the entire cell membrane perfectly at -70 mV during the high frequency EPSCs evoked by presynaptic
APs. To minimize the NMDA receptor activation, two approaches were applied. One was to position the stimulating electrode close to recorded cell soma with parameters described in methods section. The other was to lower the access resistance during the recording to improve the spatial clamping. Cells with initial access resistance higher than 30 ΩM were not continued. Cells with access resistance fluctuation larger than 15% during pre-TBS baseline or 40% during post-TBS recording were discarded. After the 100 sec TBS conditioning, we observed a consistently longer depression immediately with less variation after the conditioning (depression period: 19±1.4 min, N=11,). The longest depression period in this group was 27 min. Interestingly, no potentiation was induced with this 100-sec long TBS protocol with −70 mV command holding (conditioned synaptic strength: −14.5±10.3%, N=11, P>0.05 for comparison with baseline prior to stimulation using paired student t-test).

**Figure 2.4:** Short-term depression induced with 100 sec theta burst stimulation in hippocampal slices. The plot on the left is an example showing actual EPSC peaks induced with ‘minimal stimulation’. The arrow indicates the time the theta burst train was delivered. The noticeable level of EPSC amplitude during the initial part of the depression period after the conditioning reflects the averaged noise level during the recording. The pooled data shown on the right are from a total of 10 neurons.

This post-conditioning depression induced by the prolonged TBS protocol prompts a number of questions with regards to its mechanism. 1) It is possible that the long stimulation imposed some destructive effect on afferent fibers that innervate the recorded
2.4.2 Conduction failure does not play a significant role in our observation

Since we used minimal stimulation to generate a mixture of success and failure of evoked EPSCs, it is necessary to provide proof that failed EPSCs are not a result in conduction failure, in which the stimulated Schaffer-collateral fibers fail to convey AP due to the low level of stimulation intensity. Previously Collingridge group developed a clever way to prove whether there is conduction failure with minimal stimulation experiments (Isaac et al. 1998). The rationale is as such: for a paired-pulse protocol with interval ranges around 50-100 ms that is designed to observe paired-pulse facilitation (PPF), averaged amplitude of the second EPSC response following a successful EPSC response to the first stimulus should be comparable to that following a failed EPSC response to the first stimulus. One assumption here based upon the previous study is that each AP should generate the same amount of PPF regardless there is a successful release in response to the first AP since the facilitation is a result of the residual calcium in presynaptic terminals via VGCCs (Kamiya and Zucker 1994). Therefore, if there is indeed fiber conduction failure when the stimulus is too weak to generate an AP, or the fired AP can not travel to the presynaptic terminal, the 2\textsuperscript{nd} EPSC response following a failed EPSC response to the first stimulus is expected to be smaller than that following a successful EPSC response to the first stimulus due to lack of PPF.

To test this in our experiment, we performed five experiments with paired pulses as test stimuli throughout the experiment, before and after the conditioning. Only the 10 min pre-conditioning baseline responses were used for analysis. The interval between the test stimuli was 20 sec. Therefore, there are 30 paired stimuli in each cell recorded. However, only the EPSC responses with confirmed success or failure were used for comparison. Some of the small responses that are hard to be distinguished from noise were not used for this particular analysis. For purpose of analysis, the amplitude of the 2\textsuperscript{nd} EPSC following either a success or a failure in response to the first stimulus was extracted from the raw traces and normalized to the average of the 2\textsuperscript{nd} EPSC peak following the
successful response to the first stimulus. For clarity, we give simplified terms to describe
the EPSC response to the second stimulus either after success or failure in the first EPSC
in response to the first stimulus. 2nd peak (or EPSC)/success (or failure) is formatted to
represent the second EPSC peak or response after a successful (or a failed) EPSC
response to the first stimulus in the paired pulse. 2nd peak (or EPSC)/all is formatted to
represent the second EPSC peak or response from all the traces including success and
failure in the first EPSC response. To avoid bias, 5 consecutive cells in two consecutive
experiments were chosen for this analysis. In each cell, we took for analysis only the initial
10 min baseline before conditioning, which normally contains 30 pairs of stimuli. As shown
in Figure 5A, the paired pulse stimulation resulted in a marked paired-pulse facilitation.
The average of the first EPSC peak in the pair was 12.0±1.6 pA and the average of the 2nd
peak/all was 25.0±1.59 pA. The paired-pulse facilitation showed a 2.1- fold increase in the
averaged amplitude of 2nd EPSC/all compared to the first response (P<0.001, number of
test=130 from five cells). The 2nd peak/success was 27.4±1.96 pA (number of test=62 in
cells). The 2nd peak/failure was 22.96±2.37 pA (number of test=72 in five cells). For
comparison, averaged peaks were normalized to the peak amplitude assembly (including
all the successes and failures) to the first stimulus and were summarized in Figure 5B. No
significance was obtained between 2nd peak/success and 2nd peak/failure (P=0.16),
indicating that there is no fiber conduction failure during normal stimulation prior to high
frequency conditioning with our minimal stimulation intensity.
For traces with a success in the first response, averaged amplitudes of the two EPSC
response in the pair were almost identical (the first EPSC is 26.46±1.8 pA, compared to
the second EPSC=27.4±1.96 pA; P=0.73), consistent with the conventional notion that the
short-term facilitation exhibited in the paired-pulse protocol reflects a reduction in release
probability instead of a change in quantal content (number of vesicles) or quantal size
(size of a vesicle).
Figure 2.5: Failure analysis demonstrating unaltered peak amplitude of the second EPSC after a failed response to the first stimulus, compared to that after the successful response to the first stimulus. A, Example traces from one cell showing similarity in the peak amplitude of the second EPSC in the pair between failure and success in the first EPSC. The paired pulse interval is 50 ms. The blue trace labeled with ‘1’ is the assembly of all the traces, displaying a marked paired-pulse facilitation. ‘2’ and ‘3’ are confirmed successful and failed response averages to the first stimulus, respectively. The stimulus artifacts are removed digitally in Clampfit software with the ‘force value’ function. B, Group data for the failure analysis as exemplified shown in A. The first bar is the averaged peak amplitude of all the responses to the first stimulus, regardless success or failure and is taken as the standard of the normalization. The rest of bars on the right are the average peak EPSC
amplitude to the second stimulus in the pair either after the first EPSC response of all traces, or after a success in the first response or a failure in the first response, respectively. No difference is obtained from these three groups, analyzed with one-way ANOVA with Bonferroni post-hoc testing. All five cells are analyzed.

Having established that there is no fiber conduction failure prior to the high frequency conditioning, there is another concern that the prolonged high frequency stimulation may cause some intrinsic functional damage to the afferent fibers, which may take the same period of time to recover from the damage as the observed depression period. To rule out this possibility, we recorded the back-propagating APs from CA3 pyramidal neurons while stimulating the same region in Schaffer-Collateral pathway that is used for plasticity experiments with TBS protocols. In this experiment, current clamp recording was performed to record the membrane potential and its corresponding change. Based upon the fact that the presynaptic release is probabilistic, there is normally a wide window of stimulation intensity to generate a well-mixed success and failure for recording a voltage-clamp EPSC. For current-clamp experiments, we have attempted to obtain a stimulation threshold for firing an AP. However, the range of stimulus threshold is so narrow that we either got no failure or fail to generate any AP. This nature of stimulation threshold for firing AP is consistent with our failure analysis result suggesting that the mixture of success and failure of evoked EPSCs reflects the probabilistic nature of synaptic vesicle release, instead of fiber conduction failure. To best approximate the scheme used in voltage-clamp experiments, the threshold intensity for firing an AP was first determined. Then stimulation intensity was increased by 10% of threshold intensity. With this ‘minimal stimulation’ paradigm, a 5 min baseline was first acquired with 1 min inter-pulse intervals and an AP was induced with each stimulus prior to the TBS conditioning (100% success rate). A mixture of glutamatergic receptor antagonists CNQX (10 μM) and DL-(2R)-amino- 5-phosphonovaleric acid (DL-APV) (100 μM) was applied to the bath from the beginning of the experiment, in order to completely preclude the synaptically-induced depolarization. In addition, the signals were confirmed as APs with perfusion of 0.5 μM Tetrodotoxin (TTX). Among the three neurons tested with TTX application, APs were shut down consistently within 2 min after the start of the perfusion, that took normally less than 2 min to allow test dye to get to the recording reservoir. Unlike what was observed in voltage-clamp where a mixture of success and failure is obtained, there were consistent 100% firing in response to each stimulus pulse (Figure 6).
Immediately after delivery of 100 sec TBS conditioning, the post-conditioning recording was allowed to run for at least 40 min. For the four cells recorded, they all exhibited 100% firing rate after the 100 sec TBS conditioning with no failure. We also have two cells with only synaptically activated APs that exhibited normal sensitivity to the 100 sec TBS conditioning, with AP firing failure in the first few minutes after the stimulation (data not shown). Our result suggests that the observed depression following the 100 sec TBS conditioning is not a result of fiber conduction failure. Instead, it possibly reflects a change in the intrinsic property, for instance, a temporary change in release property.

**Figure 2.6:** Current-clamp recording on CA3 neurons showing the faithful action potential response after the 100 sec TBS conditioning directly to neuronal axons.

A, 10 μM CNQX and 100 μM DL-APV were started to wash in at the beginning of the post-TBS recording. 0.5 μM TTX was washed in approximately 5 min after delivery of the TBS. Both the pre-conditioning baseline and post-conditioning recording have 20 sec time interval between any two adjacent test pulses. The 100 sec TBS was shown in black traces with the same time scale as baseline plot. Inset shows one back-propagated AP. B, The initial 4 sec and the last 4 sec of the 100 sec TBS-induced EPSP responses were taken out to provide a more detailed behavior of the eEPSP responses. C, Schematic plot for the arrangement of recording and stimulation electrodes.
2.4.3 Localization of the depression induced by prolonged TBS conditioning

There has been a general, although not conclusive, agreement that a change in frequency of miniature or spontaneous EPSCs reflects changes in presynaptic vesicle release probability while a change in amplitude of EPSCs tends to reflect the change in postsynaptic receptors (Prange and Murphy 1999; Redman 1990). However, discovery of silent synapses and their contribution to LTP provides evidence for exceptions to the convention above-mentioned (Isaac et al. 1995; Liao et al. 1995). LTP can be accompanied by a decreased failure of synaptic transmission from conversion of silent synapses into functional synapses, possibly by addition of new AMPA receptors to the stimulated synapses (Shi et al. 1999). For our case, we can not simply rule out the possibility that the observed depression results from loss of functional synapses with prolonged stimulation. One phenomenon that can help to identify the locus of an observed synaptic change is the simultaneous observation of AMPA and NMDA receptor-mediated synaptic responses. If the AMPA receptor-mediated synaptic response exhibits a parallel change as the NMDA receptor-mediated synaptic response, it supports a presynaptically induced change, based upon the assumption that presynaptically released transmitter would affect AMPA receptors and NMDA receptors in parallel. In contrast, if a significant discrepancy is observed between the AMPA and NMDA receptor-mediated responses, it would suggest a postsynaptically induced change. To determine the locus of the depression induced by the prolonged stimulation, we made parallel recording of both AMPA and NMDA receptor-mediated currents at different holding potentials in the same cell. In one set of experiments, the membrane potential was depolarized to +40 mV for NMDA receptor mediated EPSC recording. One second after the test stimulus was applied to the neuron, the membrane potential was ramped to +40 mV in 3 sec and then was allowed to stabilize for 3 sec before a test pulse delivery for NMDA receptor mediated-current. To reduce the likelihood that repeated depolarization paired with synaptic input will cause any significant amount of plasticity, the test frequency was reduced from 0.05 Hz (every 20 sec) to 0.017 Hz (every 60 sec). Among 3 cells recorded, two cells showed continuous deterioration in amplitude during pre-induction baseline period. It may be due to the concomitant action of ligand binding and depolarization causing a ‘long-term depression’-like response. However, among the 3 cells recorded, they all exhibited parallel change in both AMPA and NMDA receptor-mediated EPSCs.
after the 100 sec conditioning, silence followed by incomplete recovery. (more cells will be added the data pool with modified way of stimulating the NMDA receptors to minimize the transmission deterioration.)

**Figure 2.7:** parallel recording of both AMPA and NMDA eEPSC before and after the 100sec TBS conditioning.

A, An example experiment demonstrating the parallel change in EPSC peak of both AMPA (black dots) and NMDA (red dots). The arrow denotes the time the 100 sec TBS is delivered. The first pair of traces in the inset shows AMPA (bottom trace) and NMDA (upper trace) receptor-mediated EPSC averaged from all the 10 points in baseline period before the TBS conditioning. The second pair is the averaged trace averaged over the same period of time (10 min) from 30 to 40 min after the TBS conditioning. B, 3 cells are grouped showing the parallel depression of normalized peak of both AMPA and NMDA components after the 100 sec TBS conditioning. The arrow denotes the time the 100 sec TBS was delivered.

### 2.4.4 Examination of synaptic plasticity with TBS conditioning of varying length with command potential at -70 mV

With 100 sec TBS inducing a long synaptic depression, it is of interest to see how shorter or longer length of TBS conditioning would exert an impact on the synaptic transmission. If indeed the silenced synaptic transmission after the long stimulation is a
result of synaptic vesicle depletion, it would predict a loss of the depression or even the emergence of PTP if the length of TBS stimulation is shortened to a certain degree. As the presynaptic vesicle pool is assumed to be depleted by the 100 sec TBS conditioning, it is sensible to predict there will not be dramatic lengthening occurring with even longer stimulation time than 100 sec. We have adopted 4 time lengths for this purpose, 5 sec, 10 sec, 100 sec and 300 sec and plotted them as in Figure 8.

Figure 2.8: TBS of varying lengths induces variable degree of post-TBS depression.

A-D, TBS protocols of 5 sec, 10 sec, 100 sec and 300 sec induced distinct durations of post-TBS depression. For all the experiments for this figure, the membrane potential during the TBS was commanded to -70 mV. E. Group data showing the all experiments in A-D. F. The same plotting scheme with error bars spared for visual clarity. The arrow denotes the time the TBS protocol is delivered. In E and F, the values are expressed as Mean±S.E..

As shown in Figure 8, shorter conditioning protocols (5 sec and 10 sec) do not exhibit the depression typically seen with 100 sec TBS. Instead, there is a tendency of PTP emergence with the shorter conditioning protocols although in the group data this tendency has not reached a statistically significant level with the number of cells available.
For longer stimulation (300 sec), the depression period is not statistically different from that obtained with 100 sec TBS protocol (21.0±3.0 min, N=7, P>0.5). This is consistent with the speculation that the 100 sec TBS protocol is sufficiently long for depletion of both the RRP and the RP vesicles and longer TBS stimulation than 100 sec would not cause any further depression.

### 2.4.5 LTP is accompanied by a shorter depression period following the 100 sec TBS stimulation

In the first two sections of the results, we have described that the 100 sec TBS conditioning induced a long synaptic depression when amplitude of eEPSCs recovered back to baseline levels with membrane potential was commanded to -70 mV during the conditioning time, and also induced synaptic depression followed by LTP of eEPSCs when the holding potential was 0 mV during the conditioning. Once we provided the proof that the observed depression was not a result of tissue damage from a ‘destructive’ effect of the prolonged stimulation protocol, it is necessary to show that the observed depression is subject to changes in different situations. In addition to changes resulting from varying lengths of TBS protocol, another interesting finding is that LTP is accompanied by a shorter depression period compared to that obtained with 0 mV holding potential during the 100 sec TBS delivery. The depression period in LTP experiments was 5.7±1.8 min (N=7), significantly shorter than that in experiments with -70 mV holding during the TBS conditioning (P<0.001). In addition the depression level is also lessened in LTP experiment. The first 5 min eEPSCs peak averages were compared between the two groups. In control group with -70 mV during 100 TBS conditioning, the average of normalized eEPSC peaks is 0.19±0.01 while the LTP group exhibits a significantly lessened depression with the first 5min average of normalized eEPSC peaks of 1.06±0.14 (P<0.001).
2.4.6 Differential effect of high frequency versus low frequency stimulation upon synaptic transmission

A previous study from our laboratory has provided evidence that recovery from vesicle depletion is less than 20 sec (Otsu et al. 2004) after depletion by application of hypertonic sucrose. Application of hypertonic sucrose has been traditionally used for observation of depletion of RRP (Rosenmund and Stevens 1996). In non-hippocampal tissue for instance, neuromuscular junction, there was a decreasing amplitude of end-plate potential and increasing variation in the amplitude as the prolonged stimulation progressed, which has been attributed to presynaptic vesicle depletion (Glavinovic 1995). However, CA3-CA1 synapses in the hippocampus have much distinct release property with a lower release probability compared to that of the neuromuscular junction (Rosenmund et al. 1993). Therefore, we investigated whether the observed depression caused by the 100 sec TBS conditioning can be also attributed to depletion of presynaptic vesicles. The parallel depression of AMPA receptor and NMDA receptor-mediated EPSC indicates a presynaptic locus of the observed depression. With the unprecedentedly long depression that is mostly manifested in an initial silence in evoked EPSCs and length of the stimulation used, we hypothesize that both the RRP and the RP vesicles are depleted with this unique stimulation protocol. Nevertheless, we proposed an attempting
speculation that replenished vesicles first to fill the RP prior to the RRP based upon the simple evidence from previous studies that refilling the RRP takes less than a minute (Granseth et al. 2006; Otsu et al. 2004). Previous studies have shown that there is constant vesicle replenishment via recycling after batch release by high frequency stimulation (Richards et al. 2000). Therefore, vesicles would be released if replenished into the RRP, in which case the synaptic silence would not occur. To test our hypothesis, we adopted an experiment scheme used by a Japanese group (Kuromi and Kidokoro 2000). In their study, they showed that a high frequency stimulation train drove release of vesicles from the RP while the low frequency drove release of vesicles from the RRP. If this is true in our observation, we would be able to drive vesicle release from the RP with a high frequency train during the depression period assuming that there have been vesicles replenished back to the RP. To test it, we applied a 20 Hz train containing 20 pulses during the depression period at various time points after the 100 sec TBS stimulation. In a set of experiments, we performed normal experiments with the 100 sec TBS stimulation protocol and -70 mV holding command during the TBS conditioning, with 20 sec interval for test stimulation. After the TBS conditioning, a 20 Hz train with 20 pulses was delivered to the neurons at time point of 5 min, 10 min or 15 min after the TBS conditioning. Only one train was used in one recorded neuron. These data were not mixed with other data pool with the concern that mobilization of RP vesicles may affect the kinetics of replenishment of the RRP that would impose a direct effect on the depression period. As shown in Figure 10, intervention of low frequency stimulation with the 20 Hz train reveals a large discrepancy in success rate of evoked EPSC in low frequency versus high frequency stimulation, with a significantly higher success rate for high frequency stimulation. Our data support the previous data that high frequency stimulation drives release from the RP and low frequency stimulation drives release from the RRP, and provide further evidence suggesting that there is a preferential replenishment of vesicles to the RP compared to the RRP.

The differential effect of high frequency stimulation versus low frequency stimulation on driving synaptic vesicles can also serve as an additional proof that the functional integrity of either afferent fiber nor the synaptic machineries were deteriorated by the prolonged stimulation.
**Figure 2.10:** High frequency stimulation drives more consistent response than low testing frequency stimulation.

A. An example cell showing the large discrepancy in EPSC response fidelity between low testing frequency and 20 Hz train. After the TBS conditioning, the post-conditioning low frequency test recording was intervened by a 20 Hz train containing 20 pulses at time point of 5 min, 10 min and 15 min after the TBS conditioning. For this cell, only the 20 Hz train at 15 min after the TBS conditioning is shown. The holding potential during the TBS train is -70 mV. Inset shows the 20 Hz train-induced EPSCs. B. The group data showing the experiment result indicated in A. Y axis label ‘success rate’ is the ratio of EPSC success versus the total of success and failure. Asterisks denote comparison of success rate between the low frequency test EPSCs and 20 Hz EPSC train.
2.5 Discussion

In this study, we have uncovered a depression that sustained for about 20 min following a prolonged theta-burst stimulation protocol. Since it is going to be repeatedly mentioned in this thesis, a term ‘long train induced-depression’ (LTID) is given to describe this type of depression induced by the standard 100 sec TBS protocol.

There are several potential possibilities that may account for a depression following a long high frequency stimulation to a presynaptic fiber bundle. One is the physical damage to the afferent fiber resulting from sustained high frequency stimulation. We think it is quite unlikely with the type of stimulating electrode we chose (glass pipette containing ACSF as conducting solution) providing moderate current density with high impedance. Another support can be seen from unproportionately high success rate during the LTID. Another typical factor that can depression synaptic transmission is receptor desensitization normally seen for AMPA receptors. However, the time course of AMPA receptor desensitization does not match the LTID we observed. In addition, after desensitization there is normally a residual current that can be detected, unlike the total annihilation of synaptic transmission as in our case. We propose this is a result of presynaptic depletion of vesicles. To confirm this, we performed the parallel recording of AMPA and NMDA receptor-mediated current and observed a paralleled abolishment of both type of currents, supporting the presynaptic nature of the LTID.

A salient feature of the observed LTID is the atypical length that has not been reported from data obtained from the hippocampus, neither from other tissues. If the depression is a result of vesicle pool depletion, then the question that follows becomes which pool is responsible. In presynaptic matrix, there are two vesicle pools, the readily-releasable pool (RRP) and the reserve pool (RP). Normally a brief train of high frequency stimulation or a bout of hypertonic sucrose stimulation lead to depletion of only the RRP. The transmission recovery from this type of depletion is rapid (Otsu et al. 2004; Schneggenburger et al. 1999). The prolonged complete transmission silence exceeds by many folds the time required for replenishment of the RRP and therefore may reflect a depletion of both vesicle pools. Since transmitter reuptake and endocytosis is a non-stop process provided that the RRP is depleted, the prolonged silence suggests that the replenished vesicles do not go to the RRP, or at least do not become immediately releasable, indicating a preferential replenishment to the RP in contrast to the RRP. Although this represents a rather primitive and challenging hypothesis and awaits to be confirmed with further experiments, it is consistent with some physiological scenarios.
First is the size of the RP that normally constitutes 80~90% of total presynaptic vesicles (Rizzoli and Betz 2005). It is reasonable to take much longer time to replenish this pool comparing to replenishment for the RRP. Secondly, the long rest as reflected in the LTID is beneficial for neuron’s recovery from prolonged high frequency stimulation which, normally not received in either resting or active states of an animal, may represent a noxious stimulus to an animal such as epileptic activity seen in seizure patients. It would not be a challenging task to test this hypothesis even in the synapses that is difficult to resolve with normal microscopy as in the hippocampus CA1 region. To prove it, all one has to achieve is to visualize a partial refilling of the entire presynaptic boutton during which no transmission is evoked by low frequency stimulation.
2.6 References


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Chapter III

Investigation of Modulation of CREB Phosphorylation by Inactivation Profile of Voltage-Gated Calcium Channels

A version of this chapter will be submitted for publication after required experimental work is accomplished. Zhi Liu and Timothy H. Murphy. CREB phosphorylation induced by non-L-type voltage-gated calcium channels.
3.1 Summary

Our previous work indicates a dominant role of inactivation of heterologous VGCCs in determining total amount of calcium influx using complex current waveforms. In this study we are trying to extend the finding to examine whether a modification in the inactivation profile is sufficient to induce changes in level of phosphorylation of cyclic-AMP response element-binding protein (CREB) in primary hippocampal culture. One specific question is whether CREB phosphorylation can be induced by non-L-type VGCCs if they have greatly reduced inactivation. We overexpressed the accessory subunit of VGCCs β1B or β2A along with green fluorescence protein (GFP) into cultured hippocampal neurons with a customized calcium phosphate transfection method. The β2A subunit is known for its role in diminishing voltage-dependent inactivation of VGCCs. Using the fluorescence emitted by GFP as guidance, comparison of CREB phosphorylation intensity was made between a control group, a β1B transfected group and a β2A transfected group.

With 20 mM KCl as stimulus, the phosphorylation level of CREB was equally blocked by nifedipine, a specific L-type VGCC blocker, in both β1B and β2A transfected groups, suggesting that reduction of inactivation does not endow non-L-type VGCCs capability of CREB phosphorylation. Another question to be addressed was whether APs alone are capable of pCREB induction via L-type VGCCs. In the presence of antagonists of NMDA and AMPA receptors, trains of APs induced moderate phosphorylation of CREB protein that exhibited no sensitivity to L-type channel blocker, but displayed sensitivity to blockade of non-selective cation channel, TRP, suggesting that L-type VGCCs are not necessarily involved in induction of CREB phosphorylation by AP trains. In another set of experiments using GABAergic antagonists as stimulant, CREB phosphorylation showed sensitivity to NMDA receptor blocker APV but not L-type channel blocker nifedipine, providing a control for neurons’ sensitivity of CREB phosphorylation to calcium channel blockers. To summarize, 1) moderate KCl stimulation induced a strong CREB phosphorylation that was sensitive to L-type VGCC antagonism; 2) CREB phosphorylation can be induced by disinhibition that was perhaps mediated by NMDA receptors but not L-type VGCCs; 3) AP trains induced by field stimulation induced CREB phosphorylation that was only sensitive to the TRP channel blocker. Overall, our results do not suffice to either prove or reject the proposed hypothesis.
3.2 Introduction

The central nervous system (CNS) undergoes a wide variety of neuronal activities that constantly modify the strength of synaptic transmission and reshape the neuronal connectivity that requires new protein to be synthesized. Among the processes of construction of the CNS, CREB has proven to be an essential element that orchestrates most of the protein synthesis. CREB is a member of a family (CREB/ATF) of structurally related transcription factors that bind to promoter CRE sites. Activation of CREB is realized by phosphorylation of ser133 position of the protein (Gonzalez and Montminy 1989). There seems to be evolutionary development in CREB activation processes. In invertebrates like *drosophila*, CREB activation is critically dependent upon activation of protein kinase A (PKA) pathway by a wide range of stimuli (Bacskai et al. 1993; Hammer and Menzel 1995). In vertebrate animals, calcium is indicated to be critical in activation of CREB as reflected in the observation that calmodulin kinase (CaMK) inhibitor blocked synaptically driven CREB phosphorylation (Bito et al. 1996; Deisseroth et al. 1996). In hippocampal cultures, Deisseroth et al showed that CREB phosphorylation depended upon the stimulation of synaptically activated NMDA receptors, but not APs alone (Deisseroth et al. 1996; Deisseroth et al. 1998). However, contrasting evidence demonstrated that AP trains alone can back-fire hippocampal neurons in acute slices and lead to phosphorylation of CREB protein (Dudek and Fields 2002). Thus induced CREB phosphorylation was blocked by L-type calcium channel specific blocker, consistent with previous statement that L-type VGCCs are specifically associated with signaling cascades that lead to gene expression (Bading et al. 1993; Murphy et al. 1991). In this study we sought to address the question of whether non-L-type VGCCs might contribute to nuclear activity such as CREB phosphorylation in certain circumstances. Our previous study showed that inactivation played an dominant role in shaping the overall calcium influx (Liu et al. 2003). Our data also demonstrated a several fold larger overall calcium current mediated by heterologously expressed L-type VGCCs than those mediated by P/Q- and N-type channels, perhaps reflecting their larger single conductance for L-type VGCCs (~25 pS) compared to non-L-type channels (Fox et al. 1987; Plummer et al. 1989). It is likely the total amount of calcium influx in response to a depolarizing stimulus is important for L-type VGCCs’ role in inducing gene expression. If this is true, non-L-type VGCCs might contribute to gene expression to a detectable level if overall calcium influx can be made larger by certain manipulations. An effective approach to increase overall calcium influx via VGCCs is to reduce inactivation of the channels, as shown in our previous study.
in which β2A subunit largely abolished voltage-dependent inactivation for P/Q- and N-type VGCCs, but not L-type channels (Liu et al. 2003). Inspired by this evidence, we sought to test the possibility that reduced inactivation of non-L-type VGCCs with incorporation of heterologous β2A subunit contributes to CREB phosphorylation which could be then detected with immunocytochemistry. However, the result did not support our initial hypothesis. First, the KCl-induced CREB phosphorylation was not rescued with incorporation of the heterologous β2A subunit into our hippocampal cultured neurons in the presence of the L-type specific channel blocker, nifedipine (Fig 3.3). Secondly, AP trains, although successfully induced CREB phosphorylation, did not induce L-type VGCC-dependent CREB phosphorylation. Instead the enhanced pCREB level by delivery of AP trains was sensitive to non-selective cation blocker, gadolinium, perhaps reflecting an inappropriate stimulating intensity. Our results are in general inconclusive to suggest any significant role of inactivation profile of VGCCs in shaping the level of gene expression reflected in CREB phosphorylation. However, the transfection method customized for primary cultured neurons has proven to be highly efficient and reliable with negligible toxicity seen normally with this approach, perhaps recommendable to most of electrophysiological experiments as well as cytochemistry experiments.
3.3 Materials and Methods

3.3.1 Primary culture of hippocampal neurons

Primary hippocampal cultures were prepared with 18 days Wistar rat embryos. Briefly, hippocampi were dissected in Earle's Balanced Salt Solution (EBSS) (Sigma, E2888), then diced and incubated at 37°C for 15 min for digestion with the EBSS containing papain. Papain-treated cells were centrifuged and then transferred to Neurobasal Medium supplemented with 0.25% (w/v) bovine serum albumin, 0.25% (w/v) trypsin inhibitor, and 20 μg/ml DNAase to terminate the digestion. Cells were further dissociated by pipetting with a series of reducing bore-size Pasteur pipettes. Then cells were plated onto dishes bottom lined with flame-sterilized 12-mm round glass coverslips at a density of approximately 100,000 cells/ml on poly-D-lysine (MW 30,000–70,000; 200 μg/ml final)-coated dishes in Neurobasal Media supplemented with 2% B27 (Invitrogen Canada Inc.), 0.5 mM glutamine, and 10 U/ml penicillin–streptomycin in a humidified 37°C 5% CO2 environment. The maintaining media was refreshed by replacing half of the existing medium with fresh media every 4–5 days after plating and cells were used for experiments at 12 or 18 days in vitro (DIV).

3.3.2 Calcium phosphate transfection

In this study, we have modified a transfection protocol based upon a previously reported method of transfection (Kohrmann et al. 1999) and developed a customized method of transfecting primary cultured neurons with calcium phosphate. In brief, cultured hippocampal neurons were allowed to be adapted to 37°C 0% CO2 incubator for 30 min in NMEM+ solution before being added with the transfection mix containing plasmid carrying genes of interest (β2A, β1B or GFP). Then neurons were placed in 37°C 0% CO2 incubator. During the incubation time, the cell dish were checked every 15 min for crystal formation. Once the crystal was formed, transfection was terminated immediately by washing with PSB to remove calcium phosphate crystals that may harm neurons. After transfection, neurons were placed in normal incubator (37°C 5% CO2) and stayed for about two weeks before experimenting. See Figure 3.1 for a typical transfection result. Detailed method is listed in Appendix in this thesis.
Figure 3.1: Calcium phosphate transfection of primary hippocampal neurons. Example photo taken from hippocampal neurons of 13DIV transfected with GFP. Photo on the left was taken via a 20x Zeiss lens and the one on the right was from a 60x immersion lens.

3.3.3 Immunocytochemistry

Hippocampal neurons of 12-21 DIV were used for immunostaining. All immunostaining was performed in 24-well plate with neurons cultured on top of 12 mm coverslips. Before treatment, low concentration of NMDA receptor antagonist APV (20 μM) and 0.1 μM TTX were added into wells containing coverslips for 2 hours to reduce background activity. Immediately after stimulation, medium in the 24-well plate was dumped by turning the plate upside down. Fixative (4% paraformaldehyde in PBS) was added into each well with a customized suction device that equipped with 6 yellow suction tips in a row with distance between two adjacent tips the same as distance between two adjacent wells. The fixative also contained 4 mM EGTA to reduce potential unnecessary calcium influx during fixation. With this suction device, medium in entire 24 wells can be replaced in 10 sec to prevent neurons from spuriously activating CREB. 1 hour after fixing, fixative was washed out with phosphate buffered saline (PBS) and replaced with PBS containing 1:1000 antibody to Ser-133-phosphorylated CREB protein (Upstate Biotechnology). The fixed neurons were incubated in antibody-containing PBS for at least 4 hours (normally overnight). After the first antibody, antibody-containing PBS was removed and washed 5 times with normal PBS. Then a rhodamine-conjugated goat anti-rabbit antibody (Jackson Immunoresearch) with a dilution factor of 1:1000 was added.
into neurons wells and was seated for 30 min before being washed and covered on a glass slide with anti-quenching reagent fluoromount-G (Southern Biotechnology Associates, Incorporation.).

### 3.3.4 Nuclear fluorescence analysis

After immunocytochemistry, nuclear fluorescence measurement was acquired with a Zeiss Axioplan inverted microscope containing standard epifluorescence attachment and directly transferred into computer with Northern Eclipse software. Then the raw images were quantified with IMAGE-J software. In IMAGE-J software, the degree of staining intensity of nuclear phosphorylated CREB was positively associated with three factors, 1) percentage of positive staining, 2) the intensity of staining expressed as grey value in the software and 3) the area of stained nuclear. The product of the three factors was used as the formula to compare the degree of CREB phosphorylation. Since there is always some variation of phosphorylation intensity across experiments, for data consistency we set a window of averaged grey value in the control group for experiments that can proceed to analysis (averaged grey value: 45-80 taken from picture with 20x lens). Experiments with the averaged grey value outside the window were not included. All experiments were performed on different days. Comparisons between different experimental groups were made with student t-test, one-way or two-way ANOVA depending upon the number of affecting factors. All data are expressed as mean±S.E..
3.4 Results

3.4.1 Characterization of pCREB induction by KCl

To establish the level of CREB phosphorylation induced by electrical stimulation, a standard needs to be determined to make comparison across experiments. For this purpose, we first chose 90 mM KCl-containing HBSS for 1 min which is commonly used to induce maximal CREB phosphorylation (Deisseroth et al. 1998). As shown in figure 3.2, 90 mM KCl induced strong staining of phosphorylated CREB. However, the staining signal was neither fully blocked by nifedipine nor by cadmium in our preparation. To test whether there is non-selective activation of CREB phosphorylation by strong KCl stimulation, a series of KCl concentration was tested in the presence/absence of nifedipine or cadmium, all in presence of 50 μm APV and 10 μm CNQX. We found that only moderate KCl stimulation-induced CREB phosphorylation is sensitive to VGCC antagonism as shown in figure 3.2. At 20 mM and 30 mM, KCl-induced CREB phosphorylation was largely blocked by nifedipine (20 mM KCl: 1846±401, +nifedipine: 392±269, P<0.05; 30 mM KCl: 2079±180, +nifedipine: 438±222, P<0.05. Tested with paired t-test). To examine the type of neuronal activity induced by 20 mM KCl used for CREB stimulation, we performed current-clamp experiments on two hippocampal CA1 pyramidal neurons from a culture of 15 DIV (Figure 3.2B). Perfusion of 20 mM KCl-containing HBSS induced moderate depolarization with high frequency firing of APs.
Figure 3.2: L-type VGCC blocker-sensitive CREB phosphorylation is induced only by moderate KCl stimulation.

All experiments for this figure were performed in the presence of 50 μM APV in the solution. A, example of fluorescence images showing nuclear staining of phosphorylated CREB in response to a 20 mM KCl stimulation for 1 min. The horizontal bar represents 50 μm. B, example trace of AP response to 20 mM KCl application. The highest AP firing frequency is 42 Hz for firing clusters with more than 10 APs. Average frequency is 7.4 Hz for the period displayed in this figure after start of the response (about 4th AP in this figure). C, a, group data showing the nuclear CREB phosphorylation in response to varying level of extracellular KCl. N=4 (separate experiments). b, Grouped results showing effect of 20 mM KCl on the CREB phosphorylation in the presence/absence of selective L-type VGCC
3.4.2 Effect of calcium channel β subunit overexpression on CREB phosphorylation induced by 20mM KCl

Activation of the L-type calcium channels is known to be specifically linked to a group of calcium-regulated genes and increases their expression. These genes include those encoding c-Fos, brain-derived neurotrophic factor (BDNF), and Bcl-2, that are important for neuronal survival, learning, and other adaptive responses in the nervous system (West et al. 2001). Previously we have established that inactivation of VGCCs is critically important in shaping the calcium influx (Liu et al. 2003). We hypothesize that reduced inactivation may endow non-L-type VGCCs with capability to activate nuclear gene expression. Experimentally we planned to test this hypothesis by overexpressing the β2A subunit that is known to drastically reduce voltage-dependent inactivation of VGCCs, into hippocampal neurons. With this subunit incorporated into calcium channel complex, it was predicted that enhanced calcium influx via non-L-type VGCCs may lead to CREB phosphorylation in the presence of L-type channel blocker.

Introduction of extrinsic gene for β2A subunit was executed with a transfection method developed in our laboratory based upon a previous establishment for calcium phosphate transfection of primary cultured neurons (Kohrmann et al. 1999). Neurons were transfected with plasmids carrying genes encoding either GFP alone, β2A or β1B along with GFP. The average transfection efficiency is 11±2% with maximal efficiency up to 40% for a culture of 13 DIV. Under microscope, normally around 50 neurons were fluorescent in a 20x field. Coverslips with less than 20 cells (about 3% of total number of neurons) in a 20x field under microscope were not used. After stimulation with KCl, neurons were immediately fixed for immunocytochemistry. Stimulation of hippocampal CA1 pyramidal neurons with 20 mM KCl for 1 min induced a strong pCREB staining in all groups. However, the KCl-induced enhancement of pCREB staining was equally abolished by application of L-type calcium channel blocker nifedipine (50 μM) in both β2A and β1B transfection groups. Therefore the result does not support the hypothesis predicting a failure in blocking pCREB enhancement by the L-type specific blocker for neurons carrying calcium channels with newly incorporated β2A subunits.
Figure 3.3: KCl-induced CREB phosphorylation in the presence/absence of L-type VGCC blocker in hippocampal neurons

After overexpression of calcium channel subunits β2A or β1B into hippocampal neurons by calcium phosphate transfection, enhanced CREB phosphorylation by 20mM KCl stimulation was still sensitive to L-type VGCC antagonism. All experiments were performed in the presence of APV (50 μM) and CNQX (10 μM). In GFP only group, neurons were transfected with only GFP while other two grouped with either β2A or β1B gene along with GFP gene. Therefore, all fluorescent neurons carry successfully transfected calcium channel β subunit. Each datum point for the nuclear staining of phosphorylated CREB was obtained from the same coverslip for both non-fluorescent neurons and fluorescent neurons. Comparisons in the figure are made between the KCl-induced response and nifedipine block effect for either non-transfected neurons or transfected neurons. One asterisk: P<0.05; two asterisks: P<0.01. N=4. Data from this transfection group using KCl and Nifedipine are not used by other figures in this thesis.
3.4.3 Action potential-induced phosphorylation of CREB

Although β2A subunit incorporation into native calcium channels failed to rescue the KCl-induced CREB phosphorylation in the presence of L-type calcium channel blocker, it does not rule out the possibility that other types of stimuli might as well be capable of pCREB induction. It has been shown that in acute hippocampal slices, APs back-propagated along axons in the alveus traveled back to soma and induced CREB phosphorylation (Dudek and Fields 2002). To examine whether AP can sufficiently drive calcium influx via VGCCs to induce CREB phosphorylation, we made a series of stimulation patterns as shown in table 3.1 and figure 3.4 in an attempt to select an optimized frequency and pattern for pCREB induction. The stimulation paradigms included a set of uniform frequency of 1 Hz, 5 Hz, 10 Hz, 25 Hz, 50 Hz and 100 Hz. The other set of paradigm was patterned in bursts with varying bursting frequencies with the same intra-burst frequency of 50 Hz. Patterns denoted as 'Burst 1', 'Burst 2', 'Burst 3' and 'Burst 4' had their bursting frequency of 0.2 Hz, 0.4 Hz, 1 Hz and 2 Hz respectively. The width of each individual pulse was 1 ms. In the case that APs alone can induce CREB phosphorylation, we will further address whether induced CREB phosphorylation is L-type channel-dependent. Experiments were performed in presence of APV and CNQX to isolate the depolarizing effect of APs from EPSPs. All experiments were performed in a customized electrical stimulation chamber with the capacity to stimulate 4 coverslips. A pair of parallel silver wires was arranged so that electrical current passed in the solution from one side to the opposite side of the chamber. To determine an appropriate stimulation intensity, neurons were incubated in cell permeable fluorescent agent, fura-3-AM, that changes fluorescence upon calcium binding. The intensity was tested with a one second of 50 Hz stimulation. Under the microscope, the stimulation intensity was gradually increased for each attempt with the one second 50 Hz stimulation until a flash of fluorescence was detected, indicating an effective stimulation output for sufficient calcium influx. Additional 5% of this threshold intensity was added on top and adopted for all the experiments. For a control pCREB immunoreactivity signal that provides both strong staining and sensitivity to L-type VGCC blocker, 20 mM KCl-containing HBSS was chosen to induce the standard pCREB staining that has been proven to be L-type calcium channel-dependent in our experiments in a previous section. In the set of experiments
searching for optimal stimulus for pCREB induction, simple 10 Hz stimulation and a theta burst frequency with 4 Hz bursting frequency ('burst 4') turned out to be most effective in inducing CREB phosphorylation compared to other stimulation patterns, although overall signals were not strong comparing to KCl-induced staining. Based upon these results, the simple 10 Hz and 'burst 4' protocols were taken for further pharmacology experiments. The pharmacology experiment included control group with no stimulation, a group with 10 Hz stimulation, a group with 'burst 4' stimulation and 20 mM KCl stimulation for a standard immunoreactivity signal, all in presence of APV and CNQX except the KCl group. 50 μM nifedipine effectively blocked effect of 20 mM KCl-induced CREB phosphorylation. However, the same dose of nifedipine failed to block increased CREB phosphorylation induced by either 10 Hz stimulation or 'burst 4' stimulation. The result was confirmed with the non-selective VGCC blocker, 100 μM cadmium (data not shown). With all synaptic transmission and VGCCs blocked, one important route that can mediate calcium influx is the ‘transient receptor potential’ (TRP) channels that has been reported to mediate calcium influx in response to pathological stimuli and some special physiological stimuli such as Brain-derived neurotrophic factor (Amaral and Pozzo-Miller 2007; Clapham 2003). TRP channels are a family of non-selective cation channels and have been involved in ion influx invoked predominantly by painful and pathological stimuli (Moran et al. 2004). The TRP channel blocker, gadolinium (Gd^{3+}) was applied to the stimulation solution at 100 μM concentration. To our surprise, Gd^{3+} significantly brought down the pCREB immunoreactivity in both 10 Hz and ‘burst 4’ groups, as well as KCl group to a moderate extent.
**Table 3.1:** Comparison of effect of varying stimulation pattern on the degree of CREB phosphorylation

<table>
<thead>
<tr>
<th>Bursting Hz</th>
<th>Ctrl</th>
<th>1 Hz</th>
<th>5 Hz</th>
<th>10 Hz</th>
<th>25 Hz</th>
<th>50 Hz</th>
<th>100 Hz</th>
<th>Burst 1</th>
<th>Burst 2</th>
<th>Burst 3</th>
<th>Burst 4</th>
<th>KCl</th>
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</thead>
<tbody>
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<td>28.5</td>
<td>39.1</td>
<td>497.3</td>
<td>140.7</td>
<td>573.8</td>
<td>62.5</td>
<td>812.7</td>
</tr>
<tr>
<td>Ctrl</td>
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<td>39.1</td>
<td>2</td>
<td>N/A</td>
<td>497.3</td>
<td>140.7</td>
<td>N/A</td>
<td>812.7</td>
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<td>62.5</td>
<td>N/A</td>
</tr>
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<td>140.7</td>
<td>3</td>
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<td>573.8</td>
<td>62.5</td>
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<td>573.8</td>
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<td>6</td>
<td>N/A</td>
</tr>
<tr>
<td>5 Hz</td>
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<td>62.5</td>
<td>6</td>
<td>N/A</td>
<td>812.7</td>
<td>187.5</td>
<td>N/A</td>
<td>812.7</td>
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<td>62.5</td>
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<tr>
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<td>812.7</td>
<td>187.5</td>
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<td>62.5</td>
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<tr>
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<td>812.7</td>
<td>187.5</td>
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<td>812.7</td>
<td>812.7</td>
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**Table 3.2:** Effect of treatments of calcium channel blockers on the degree of CREB phosphorylation

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<td>151.0</td>
</tr>
<tr>
<td>N</td>
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</tr>
</tbody>
</table>
**Figure 3.4:** APs do not appear to mediate pCREB formation via L-type VGCCs in our preparation.

Left graph shows the normalized pCREB induced by different patterns of AP train, in the presence of APV (50 μM) and CNQX (10 μM) for all groups but KCl group. The patterned stimulation was delivered with fixed number of pulses (1000 pulses), each pulse with 1 ms pulse width. Burst1-4 denote theta burst stimulation with 5 pulses in a burst and bursting frequency of 0.2 Hz, 0.4 Hz, 1 Hz and 2 Hz for Burst1, Burst2, Burst3 and Burst4 respectively. 10 Hz and higher frequency of theta burst stimulation ('Burst 4') showed stronger effect compared to other stimulation patterns although overall intensity induced by AP trains was moderate compared to 20 mM KCl-induced effect (the rightmost bar). The graph on the right demonstrates the pCREB immunoreactivity induced by 20 mM KCl and APs induced with 10 Hz and theta burst stimulation ('Burst 4' group). Synaptic activity was blocked with 50 μM APV and 10 μM CNQX in order to reveal the effect of activation of VGCCs. With 'Burst 4' stimulation, although no significance was obtained for APV+CNQX+Gd³⁺ group (P=0.058), significant differences were detected from comparison between APV+CNQX+Gd³⁺ group (P<0.05) and APV+CNQX+DMSO group as well as APV+CNQX+Nif group (P<0.05). In both graphs, all means were normalized to the 20 mM KCl-induced pCREB level with no drug application. Asterisks denote comparison to the same stimulus-induced pCREB (the leftmost bar in 10 Hz and 'burst 4' group).
3.4.4 Disinhibition-induced pCREB is mediated via synaptically induced calcium influx

One concern with stimulation of hippocampal neurons for measuring CREB phosphorylation level with either KCl or field electrical stimulation using patterned trains is that they are not normally experienced by neurons in physiological scenarios. In addition, the electrical stimulation for instance, is difficult to control for an appropriate intensity that does not lead to any physical destruction of neuronal membranes. To improve it and also to confirm the result with KCl and electrical stimulation, we decided to use short periods of disinhibitory agents, bicuculine and picrotoxin, to examine their disinhibitory effect on CREB phosphorylation and their sensitivity to antagonisms. First picrotoxin was chosen to test for an optimized period of disinhibition, with 30 sec, 1 min and 3 min of perfusion of 100 μM picrotoxin. The result indicated that with longer perfusion of picrotoxin it tended to lose sensitivity to all the antagonists applied, including APV, nifedipine and a combination of APV and nifedipine, with 3 min application of picrotoxin showing no reduction in pCREB level for any antagonist perfusion (Figure 3.5). In contrast, 30 sec perfusion of picrotoxin showed greatest sensitivity to APV block, but not to nifedipine block, suggesting a synaptically mediated pCREB enhancement. 1 min perfusion of the drug also brought down the pCREB level to an apparently lower level, but no statistical significance was obtained. Similar results were obtained in a set of experiments with a similar GABAergic transmission inhibitor bicuculine with 30 sec perfusion of the drug (see Figure 3.5).
**Table 3.3:** Experiments seeking an optimal time of perfusion with picrotoxin to induce CREB phosphorylation.

<table>
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<td>3</td>
</tr>
<tr>
<td>1 min</td>
<td>485.6</td>
<td>156.5</td>
<td>3</td>
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<tr>
<td>3 min</td>
<td>672.4</td>
<td>71.7</td>
<td>3</td>
</tr>
<tr>
<td>+APV+Nif</td>
<td></td>
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<tr>
<td>30 sec</td>
<td>17.0</td>
<td>26.1</td>
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<tr>
<td>1 min</td>
<td>135.8</td>
<td>52.2</td>
<td>2</td>
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<tr>
<td>3 min</td>
<td>560.3</td>
<td>104.3</td>
<td>3</td>
</tr>
</tbody>
</table>

**Table 3.4:** Experiments showing the sensitivity of CREB phosphorylation induced by GABAergic antagonists to different calcium channel blockers.

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>SE</th>
</tr>
</thead>
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<tr>
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<td>Picrotoxin</td>
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<tr>
<td>Picrotoxin</td>
<td>63.0</td>
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</table>
Figure 3.5: Disinhibition induced pCREB formation may be mediated by synaptic NMDA receptors.

Bar graph on the left demonstrates CREB phosphorylation induced by picrotoxin (100 μM) and its effect in the presence of APV (50 μM) or nifedipine (50 μM) or a combination of APV and nifedipine, with periods of 30 sec, 1 min or 3 min. Comparisons are made between mean intensity of pCREB induced by picrotoxin in the presence and absence of antagonist. Data were acquired from four experiments from different days. Note that the picrotoxin effect upon pCREB is only inhibited by APV but not by nifedipine. The bar graph on the right describes a parallel experiments with bicuculine (20 μM) and picrotoxin (50 μM) with a scheme similar to the left graph with 30 sec drug perfusion time adopted from experiments shown from the left graph. There is no data overlap between two groups. Comparisons are made between mean pCREB immunoreactivities of bicuculine/picrotoxin alone group and the group with their corresponding blocker. The right graph was obtained from five experiments acquired from different days. One asterisk: P<0.05; Two asterisks: P<0.01; Three asterisks: P<0.001.
3.5 Discussion

Our results have demonstrated that overexpression of the VGCC β2A subunit failed to rescue the CREB phosphorylation induced by KCl in the presence of L-type VGCC blocker. This is in apparent contrast to our hypothesis that emphasizes the possibility that reduced inactivation may enable non-L-type VGCCs to compensate the reduced CREB phosphorylation invoked by incorporation of β2A subunit. The difference might be accounted by a number of explanations. The first possibility is that the neuronal activity induced by high KCl is not significantly influenced by a change in the inactivation profile of local VGCCs. As shown in figure 3.2, 20 mM KCl-containing HBSS induced two components of membrane potential deflection. One is a moderate depolarization as a result of reduced driving force for K⁺ at rest. The other is the AP firing that was induced in our preparation by suprathreshold depolarization. In the two cells recorded in current-clamp mode, neurons started to fire trains of APs before the membrane depolarized by 10 mV which is far below the normal AP firing threshold and is below the level required for VGCC activation based upon the normal current-voltage relationship of VGCCs. It is possible that the high frequency firing is an indirect result from disinhibition of neighboring interneurons or glia. However this possibility awaits proof. Although APs by themselves are too short to be affected by inactivation the time course of which is much longer than that of an AP, a high frequency AP train may induce varying degrees of inactivation of VGCCs depending upon the sustained depolarization level upon which the AP train is seated (Liu et al. 2003). In our case, the moderate depolarization level upon which the AP firing train was seated may be negligible and not cause any significant inactivation of VGCCs during the AP train. This may explain the failure of β2A subunit transfection in influencing the pCREB dependency upon L-type VGCCs. Another uncertainty is that β2A subunit may not be bound to different calcium channel α1 subunits with equal affinity. Although we have shown in our previous study that α1C, α1B and α1A can all form heterologous calcium channels in HEK cells (Liu et al. 2003), evidence for stoichiometry of native calcium channel complexes is still scarce. In our hippocampal preparation, we can not rule out the possibility that α1 subunits in non-L-type calcium channels may have low affinity for overexpressed β2A subunit, although native β2A subunit expression in the hippocampus is normally insignificant (Ludwig et al. 1997). AP trains in different patterns were applied as an alternative approach after experiments with KCl as stimuli for pCREB induction. There have been different opinions with regard to
the capability of APs as an effective stimuli by themselves for induction of CREB phosphorylation. Tsien group showed that high frequency AP train failed to induce CREB phosphorylation (Deisseroth et al. 1996; Deisseroth et al. 1998). In contrast, another study demonstrated that stimulation of hippocampal CA1 neuron axons backfired the soma and induced in situ phosphorylation of CREB protein, providing evidence for AP alone as a sufficient induction stimulus for CREB phosphorylation (Dudek and Fields 2002). We think the study by Dudek et al presented a more physiological setting for investigation of the role of AP in eliciting CREB phosphorylation, with the direct AP invasion from axon to soma. However we have scrutinized the factors in Tsien’s group study that may influence the success of an AP train in eliciting CREB phosphorylation. We have designed a series of AP trains in different frequencies and patterns in an attempt to find an optimized stimulation paradigm for CREB phosphorylation. Our results showed that simple 10 Hz and a theta-burst stimulation with 4 Hz bursting frequency had the greatest effect on eliciting CREB phosphorylation. However, to our surprise, their effect was not sensitive to L-type calcium channel block. Instead thus induced CREB phosphorylation was blocked by non-selective cation channel blocker Gd\(^{3+}\). One possibility is that a direct passing of an electrical current over the surface of neuron layer in cell chamber may cause certain degree of physical damage to the cell membrane. We think passing electrical current through entire cell medium as in our case is at an apparent disadvantage comparing to focal stimulation to axons in the Alveus (Dudek and Fields 2002), although we have made a moderate adjustment (a 5% over threshold intensity) for the stimulating intensity. Activation of TRP channels may be a reflection of this pathological action. Future direction for a convincing conclusion may lie in the establishment of an experiment setting that allows for both transfection of heterologous calcium channel subunit and unharmful stimulation that can be obtained by using microisland culture or by dual recording on two interconnected neurons simultaneously.
3.6 References


Chapter IV

Cholinergic Modulation of GABAergic Activity in the Striatum

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A version of this chapter has been published in J.Neurophysiol.98(2):581-93 (2007) by Zhi Liu, Yo Otsu, Cristina Vasuta, Hiroyuki Nawa and Timothy H. Murphy, as “Action potential independent GABAergic tone mediated by nicotinic stimulation of immature striatal miniature synaptic transmission”.

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4.1 Summary

Stimulation of presynaptic nicotinic acetylcholine receptors (nAChRs) increases the frequency of miniature excitatory synaptic activity (mEPSCs) to a point where they can promote cell firing in hippocampal CA3 neurons. We have evaluated whether nicotine regulation of miniature synaptic activity can be extended to inhibitory transmission onto striatal medium spiny projection neurons (MSNs) in acute brain slices. Bath application of micromolar nicotine typically induced 12-fold increases in the frequency of miniature inhibitory synaptic currents (mIPSCs). Little effect was observed on the amplitude of mIPSCs or mEPSCs under these conditions. Nicotine stimulation of mIPSCs was dependent on entry of extracellular calcium since removal of calcium from perfusate was able to block its action. To assess the potential physiological significance of the nicotine-stimulated increase in mIPSC frequency, we also examined the nicotine effect on evoked IPSCs (eIPSCs). eIPSCs were markedly attenuated by nicotine. This effect could be attributed to two potential mechanisms: transmitter depletion due to extremely high mIPSC rates and/or a reduction in presynaptic excitability associated with nicotinic depolarization. Treatment with low concentrations of K+ was able to in part mimic nicotine’s stimulatory effect on mIPSCs and inhibitory effect on eIPSCs. Current-clamp recordings confirmed a direct depolarizing action of nicotine that could dampen eIPSC activity leading to a switch to striatal inhibitory synaptic transmission mediated by tonic mIPSCs.
4.2 Introduction

Miniature transmitter release results from the constitutive low-level release of individual vesicles of neurotransmitter. Since the 1950s this form of synaptic transmission was thought to be a reflection of a leaky evoked release mechanism and it was not clear whether it had a function of its own (Otsu and Murphy 2003). Previous studies suggest that miniature release (mini) can reflect both the local chemistry of synapses (Murphy et al. 1994) as well as the network properties of neurons (Carter and Regehr 2002; Sharma and Vijayaraghavan 2003). Although mini rates are typically low, recent data describe how nicotine stimulation of nicotinic acetylcholine receptors (nAChRs) can elevate the frequency of excitatory glutamatergic minis to levels that can affect network behavior (Sharma and Vijayaraghavan 2003).

Acetylcholine (ACh) differs from other neuromodulators such as dopamine (DA), noradrenaline and metabotropic glutamate receptor agonists, in that it can affect presynaptic calcium levels through direct calcium influx via nAChRs and/or activation of VGCCs resulting from depolarization induced by Na$^+$ influx through nAChRs (Dani 2001; Gray et al. 1996). This nAChR-mediated increase in presynaptic calcium is then associated with the increase in minis (Gray et al. 1996; Guo et al. 1998; Kiyosawa et al. 2001; Lena and Changeux 1997; Sharma and Vijayaraghavan 2003). In striatum, multiple nAChR subunits are expressed (Champtiaux et al. 2003; Wada et al. 1989; Zoli et al. 2002) and evidence suggest that presynaptic nAChRs regulate the secretion of DA from substantia nigra DAergic terminals (Champtiaux et al. 2003; Marshall et al. 1997; Zhou et al. 2001). However, relatively little is known about the effect of nicotine on the release of fast transmitters such as glutamate and GABA in the striatum (Kita 1996; Koos and Tepper 2002; Misgeld et al. 1980).

About 90% of total striatal neurons are medium spiny neurons (MSNs) that employ GABA as a neurotransmitter and project to other regions such as substantia nigra pars reticulata and globus pallidus. MSNs receive inhibitory and excitatory afferents predominantly from GABAergic interneurons and from the cerebral cortex and thalamus, respectively (For review, see Wilson, 2004). Connections between MSNs are rare (Koos et al. 2004), especially in mature striatum, although recently more direct evidence with dual patch-clamp recording have demonstrated connections between MSNs in ventral striatum of relatively mature brains (Taverna et al. 2004). It is possible that GABAergic or glutamatergic transmission onto MSNs is presynaptically modulated by ACh released from cholinergic interneurons. Given the existence of nAChRs on presynaptic terminals
that innervate MSNs and the nAChRs’ unique ability to increase presynaptic calcium concentration, we determined whether miniature synaptic activity in the striatum might be regulated by this mechanism. We report that nicotine can produce almost 12-fold increases in striatal GABAergic mIPSCs, but not glutamatergic mEPSCs through a mechanism associated with calcium entry through calcium permeable nicotinic receptors. These high rates of minis stimulated by nicotine dampen evoked IPSCs (eIPSCs), suggesting a transition to a mode of signaling involving tonic mIPSC-mediated inhibition.
4.3 Materials and Methods

4.3.1 Slice preparation

Wistar rats (postnatal day 8-15) were anaesthetized with halothane and decapitated. All animals used in this project were cared for in accordance with regulations of the Canadian Council on Animal Care. Coronal slices of striatum (250-300 μm thickness) were prepared in an ice-cold modified artificial cerebrospinal fluid (ACSF) and incubated at room temperature for at least 1h in normal ACSF. The composition of normal ACSF was as follows (in mM): 119 NaCl, 2.5 KCl, 2.5 CaCl₂, 1.3 MgSO₄, 1.0 NaH₂PO₄, 26.2 NaHCO₃, 11 glucose (pH 7.4 with 95 % O₂ / 5 % CO₂). In the modified ACSF, NaCl was substituted for 200 mM sucrose, and the solution contained 0.8 mM CaCl₂, 4 mM MgSO₄ and 1 mM kynurenate.

4.3.2 Electrophysiology

MSNs in striatum were visualized using a water immersion objective lens (Olympus 60X) and were identified by shape and size (ovoid cell body with 8-14 μm major axis). A whole-cell patch electrode (~4 MΩ) was used to record synaptic responses from these neurons in a voltage- or current-clamp mode with an Axopatch 200B. Series resistance was compensated 60-70 %. The cells were clamped at 0 mV or -65 mV to record inhibitory synaptic currents or excitatory synaptic currents, respectively. To record miniature inhibitory synaptic currents (mIPSCs) the recording solution included 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (10 μM), D,L-2-amino-5-phosphonovaleric acid (APV) (100 μM) and tetrodotoxin (TTX) (1 μM). Spontaneous and evoked IPSCs (sIPSCs and eIPSCs) were recorded in the absence of TTX. A bipolar tungsten electrode was put in the striatum (~200 μm from a recording cell) to induce eIPSCs. Current pulses were delivered through the electrode for 150 μs at a range from 100 to 500 μA (1.1 to 1.8 times higher than threshold intensity). To record miniature excitatory synaptic currents (mEPSCs), bicuculline methiodide (BMI) (20 μM) and 1 μM TTX were added to the recording solution. For voltage-clamp recording, the routinely used the internal solution contained (in mM): 123 Cs-methane sulfonate, 10 HEPES, 7.5 CsCl, 0.2 EGTA, 8 NaCl, 4 MgATP, 0.3 Na₂GTP, 5 QX314-Cl and 5 Biocytin-Cl. In one set of experiments where constant negative holding potentials were used to assess mIPSCs as inward currents we used a modified internal solution of the following composition (in mM): 130 CsCl, 10...
HEPES, 0.2 EGTA, 4 MgATP, 0.3 Na₂GTP, 5 QX314-Cl and 5 Biocytin-Cl. The pH of the two internal solutions was adjusted to 7.2 by CsOH and the osmolarity was adjusted to 290 mOsm. The liquid junction potential was not corrected. When responses were monitored with current-clamp mode, the composition of internal solution was changed as follows (mM): 129.4 K-gluconate, 10 HEPES, 11.1 KCl, 0.02 EGTA, 4 NaCl, 3 MgATP, 0.3 Na₂GTP (pH adjusted to 7.25 using KOH). To set the equilibrium potential for chloride to –58 mV as observed in MSNs (Jiang and North 1991), the recording bath solution was changed as follows (mM): 125 NaCl, 2.5 KCl, 2.5 CaCl₂, 1.5 MgCl₂, 1.25 NaH₂PO₄, 26 NaHCO₃, 9 glucose (pH 7.4 with 95% O₂/ 5% CO₂) (Koos and Tepper 1999). Recordings were all performed at room temperature. Nicotine was applied with a local perfusion system equipped with a Y-shape tube or bath application. Various drugs to modify effects of nicotine were applied at least 10 min before agonist treatment. In the case of bath application, it takes about 2 min to reach drug solution into the recording chamber from its reservoir. In figures time zero indicates the time at which the reservoir was changed, not the time of bath equilibration. Signals were digitized at 5 kHz and filtered at 2 kHz (low-pass Bessel filter). Drugs used were obtained from the following sources: TTX, nicotine tertrate, APV, BMI, mecamylamine (MEC), methyllycaconitine (MLA) and SKF38393 from Sigma; CNQX from Tocris; QX-314-Cl from Alomone labs; (+) SCH23390 and (-) sulpiride from RBI.

4.3.3 Histology

After recording, some slices were randomly chosen for biocytin-staining to confirm that the recorded cells possess the characteristic morphology of MSNs. Slices with biocytin-injected cells were fixed with 4% paraformaldehyde in 0.1M phosphate buffer (PB) for more than 2 hrs at 4°C and soaked in 30% sucrose in phosphate buffered saline (PBS) for another 12 hrs. The tissue was frozen with dry ice and thawed twice, and then incubated for 30 min in PBS containing 0.5% H₂O₂ and 10% ethanol at room temperature to suppress endogenous peroxidase activity. The tissues were rinsed in PBS for 1 hr and incubated in PBS containing 1% Triton X-100 (TX) for 4hrs at room temperature. After washing for 1 hr, they were incubated in a 0.5% TX in PBS containing avidin-biotin-peroxidase complex (ABC solution; ABC Elite, Vector Laboratories) for 3 hrs at room temperature. Visualization of biocytin-injected cells was achieved with 0.05M Tris-HCl buffer (pH 7.4) containing 3,3’-diaminobenzidine tetrahydrochloride (DAB; 0.05%) and 0.01% H₂O₂.
4.3.4 Data analysis

Spontaneous and miniature synaptic currents data were analyzed with AxoGraph 4 and pClamp 9 (Axon Instruments). An event was detected with a criterion of a threshold > 3×SD of baseline noise and > 3-5 pA amplitude. The detected mIPSCs were then manually inspected to exclude false events caused by an artificial source such as environmental noise. In young neurons the frequency of mIPSCs could be very low leading to massive fold changes in mIPSC frequency (over 100 fold was observed at times) following addition of nicotine. These very large fold changes in frequency made it difficult to assess the pharmacology of nicotine and its mechanism. Therefore, only cells that had a basal frequency of mIPSCs between 0.1 – 2 Hz were used to assess the pharmacology and mechanism of nicotine effects. To evaluate the change of GABAergic activity, the percentage increase in mIPSC frequency was routinely used and was determined by comparing the average number of events per bin (1 bin = 20 sec) during the last two minutes of the control period with the average during the peak of the nicotine effect in which 3 bins (before, peak, and after) were averaged. Data from cells with unstable baseline firing (15% or above fluctuation during the last 3 min of the control period) are not included. The average values are provided as mean ± S.E.M. The differences in the mean peak frequency were tested by Mann-Whitney U test.
4.4 Results

4.4.1 Effect of nicotine on membrane excitability in MSNs

The effect of nicotine on MSNs in striatal slices from 8-15 day rat pups was studied to determine whether nAChR stimulation affects their excitability. MSNs were identified by their unique electrophysiological and morphological characteristics. MSNs had a relatively hyperpolarized resting potential (-76.3±1.4 mV, N = 11), inward rectification (Figure 4.1Aa, b), and spiny dendrites (Figure 4.1Ac). These properties are consistent with previous work (Kawaguchi 1992; Kawaguchi et al. 1989; Tepper et al. 1998). To assess potential effects of nicotine on MSNs, we performed recordings in current-clamp mode to determine whether nicotine led to changes in MSN excitability. For these experiments we used intracellular Cl⁻ concentrations (15 mM) which correspond to a –58 mV reversal potential for chloride that is consistent with previously reported values from intracellular recordings (Jiang and North 1991; Koos and Tepper 1999). Addition of 20 µM nicotine via bath application under current clamp led to an increase in noise and a modest depolarization of MSNs (3.17±0.3 mV; N = 3) (Figure 4.1Ba), but APs were not induced. This increased noise was blocked in the presence of GABAergic (20 µM bicuculline methiodide (BMI)) and glutamatergic (10 µM CNQX and 100 µM APV) receptor blockers. However the modest depolarization persisted (Figure 4.1Bb). The noise was thought to be induced by an increase in GABA or glutamate release, while the depolarization could be induced by a direct postsynaptic effect of nicotine on MSNs.
Figure 4.1: Effect of nicotine on membrane potential and excitability in medium spiny neurons (MSNs)

A. Characteristic membrane properties and morphology of a medium spiny neuron (MSN). 
Aa. Traces show individual voltage responses to series of 400 ms current pulses from -100 pA with 20 pA increasing current steps. Ab. Relationship between injected current and steady-state voltage responses are plotted. Note the inward rectification of MSNs. Ac. An example of a MSN stained after recording. The scale is 10 µm. 

B. Nicotine (20 µM) directly depolarizes MSNs in current-clamp mode. 
Ba. Membrane depolarization and increased noise were observed during application of 20 µM nicotine under normal conditions. Bb. Membrane depolarization was not blocked, but increased noise was decreased in the presence of glutamate (10 µM CNQX and 100 µM APV), and GABA (20 µM BMI) receptor blockers. Although 20 µM nicotine depolarized the neurons in current-clamp mode, it failed to produce APs as the firing threshold for MSNs was positive to −35 mV. 

C. Minimal change in excitability following nicotine treatment. To assess
changes in excitability, we applied depolarizing injections of current through the recording electrode during a control period or during the application of 20 μM nicotine. 

**Ca.** An example of 20 μM nicotinic effect on membrane potential and spike latency. Nicotine depolarized the membrane potential by only a few mV, which was measured by averaging membrane potential for 3 s before current injection (filled circles) and shortened the first spike latency, which was the time between onset of current injection and the peak of the first AP (open circles). Raw traces of stimulus #10 and #20 are shown in **Cc. Cb.** During nicotine application, a small increase in the number of spikes induced by each current injection was detected. 

**Cc.** Two traces, before (#10; grey) and during nicotine (#20; black) application are superimposed. * indicates the position of AP in trace #20. Note the first spike latency is shortened and the resting membrane potential was depolarized during nicotine application. (Figure used with permission).

To assess whether nicotine altered spike threshold, MSNs were held at resting potential in current-clamp recording mode, and depolarizing pulses which induced 3-5 spikes with a 1st spike latency of 100-120 ms were injected at 0.1 Hz. Bath application of 20 μM nicotine caused membrane depolarization and a shortened 1st spike latency (113.3 ± 11.6 ms under control condition vs. 82.0 ± 3.3 ms in the presence of nicotine; N = 3) (Figure 4.1Ca, c) and a slight increase in spike number with threshold depolarizing pulses (4.1 ± 1.3 spikes under control condition vs. 5.0 ± 1.1 spikes in the presence of nicotine; N = 3 cells; Figure 4.1Cb). In summary these experiments indicated a limited effect of nicotine on the excitability of MSNs.

### 4.4.2 Effect of nicotine on miniature synaptic events

In current-clamp experiments we detected an inhibition of nicotine-induced noise by glutamatergic and GABAergic antagonists (Figure 4.1Ba, b). To determine whether this effect was induced by presynaptic GABAergic or glutamatergic activity, we monitored miniature synaptic events under voltage-clamp recording mode. Miniature inhibitory postsynaptic currents (mIPSCs) were recorded as outward currents at a holding potential of 0 mV in the presence of CNQX (10 μM), APV (100 μM) and TTX (1 μM) (Figure 4.2Aa). mIPSCs were abolished by addition of BMI (20 μM), a GABA<sub>a</sub> receptor antagonist (data not shown). Thus the pharmacological data indicated that both Y-tube (N = 8/8) or bath (N = 13/13) application of 20 μM nicotine results in a robust increase in mIPSC frequency. In
Figure 4.2A, representative traces are shown during control and after 1 min of 20 µM nicotine local application through Y-tube perfusion. Use of a semi-automated mIPSC detection system indicated an increase in mIPSC frequency greater than 10-fold (11.8±1.9 fold, N = 15 cells) with 20 µM nicotine application (Figure 4.2B). The effect of nicotine was fully reversible upon washout and was concentration-dependent as shown in our data obtained with 1 and 100 µM nicotine (Figure 4.8Bb and supplementary figure 4.1).

In contrast to effects of noradrenaline in hypothalamus (Gordon and Bains 2005), analysis of mIPSC amplitudes demonstrated that nicotinic stimulation led to an increase only in frequency and not in amplitude (Figure 4.2C, N = 9/13, P < 0.01; Kolmogorov-Smirnov (KS)-test). In a subset of neurons (4 cells) we did observe some apparent changes in amplitude. Due to the high frequency of events in some cases apparent increases in amplitude could be due to random summation of mIPSCs given their relatively long time course. In addition, the time course of mIPSCs was unaffected by nicotine treatment (Figure 4.2Ab). Nicotinic agonist effects were detected in all MSNs examined and cells with relatively low basal mIPSC frequency had the largest fold increase during nicotine treatment (Figure 4.2D). To make our pharmacological analysis more quantity reliable we did not include neurons with basal frequency less than 0.1 Hz. However, there was no significant correlation between frequency in control and nicotine treatment (Figure 4.2E).

Although 20 µM nicotine robustly elevated the frequency of mIPSCs, the effect was selective since analysis of miniature excitatory postsynaptic currents (mEPSCs) recorded at –65 mV under pharmacological blockade of GABA_A receptors (using 20 µM BMI) and 1 µM TTX indicated no significant effect of nicotine on the frequency of mEPSCs (Figure 4.3Aa, B,D). In addition to there being no apparent effect on mEPSC frequency, we did not detect any change in the mEPSC kinetics (Figure 4.3Ab) or amplitude (Figure 4.3C, N = 5/5, P > 0.1; KS-test), indicating a specific action of nicotine on inhibitory presynaptic terminals.
Figure 4.2: Nicotine increases the frequency of mIPSCs in MSNs

Aa. Whole-cell voltage-clamp recording traces are shown depicting mIPSCs (in the presence of 1 μM TTX, 100 μM APV, and 10 μM CNQX) and with addition of 20 μM nicotine through the Y tube. Holding voltage was 0 mV. Ab. Representative averaged traces (13 sweeps) showing mIPSC kinetics with (black trace) and without (grey trace) nicotine treatment. The two traces are over-plotted and normalized to the peak current for control and nicotine cases. The decay was 24.7 ms. B. Histogram showing increases in mIPSC frequency in a striatal neuron. In this cell, nicotine applied through Y-tube produces about a 5-fold increase in mIPSC frequency that reverses within minutes of washout. C. Cumulative probability histogram demonstrating mIPSC amplitude
distribution under control conditions (303 events) and with 20 μM nicotine (440 events) from the same cell in B. No significant difference in mIPSC amplitude was observed following nicotine treatment (KS-test; P = 0.44). D. Relationship between nicotinic enhancement and basal mIPSC frequency. Basal mIPSC frequency and the fold change in frequency following 20 μM nicotine treatment are plotted. Cells with relatively low basal mIPSC frequency have the largest fold increase during nicotine treatment (r = -0.68, P = 0.01). E. Peak mIPSC frequency as a function of basal mIPSC frequency. Nicotine stimulates all cells to a similar frequency of mIPSCs regardless of their basal mIPSC frequency (r = 0.096, P = 0.76). (Figure used with permission).

Figure 4.3: No significant effect of nicotine on mEPSC frequency in MSNs

Aa. Representative traces of mEPSCs recorded in the presence of 1 μM TTX and 20 μM BMI at -65 mV holding potential. In this example and group data no significant change in frequency or amplitude of mEPSCs was observed with 20 μM nicotine treatment for 2 min
through Y-tube. **Ab.** Representative average mEPSCs (23 sweeps) from control conditions (grey trace) and following treatment with 20μM nicotine (black trace). No significant difference in mEPSC kinetics was observed. **B.** Time-course analysis of nicotine effect on mEPSC frequency. **C.** Cumulative probability histogram demonstrating mIPSC amplitude distribution under control conditions (641 events) and with 20 μM nicotine (676 events), showing little effect of nicotine on mEPSC amplitude (KS-test; P = 1.00). **D.** Group data of nicotine effect on mEPSC frequency (N = 5-6 cells). mEPSC frequency in each cell is normalized to an average frequency measured over 2 min before nicotine application. There was little effect of nicotine on mEPSC frequency. (Figure used with permission).

### 4.4.3 Calcium dependency of nicotine effect on mIPSC frequency

**Figure 4.4:** Calcium-dependence of nicotinic stimulation of mIPSC frequency

Current traces from a striatal neuron in the presence of 1 μM TTX, 100 μM APV, and 10 μM CNQX at 0 mV holding potential showing the effect of nicotine under various conditions. **Aa.** Nicotine (20 μM) enhanced mIPSC frequency during bath application. **Ab.** Two traces before and 100 s after nicotinic application from **Aa** are expanded. **B.**
Pre-treatment with 0 mM extracellular calcium solution blocked the nicotine effect. C. Nicotine enhancement of mIPSC frequency is independent of VGCC activation. MSNs were treated with 20 µM nicotine in the presence or absence of a combination of CdCl2 (200 µM) and NiCl2 (1 mM). Despite the presence of saturating concentrations of VGCC blockers, nicotine still had a pronounced effect on mIPSC frequency. D. Average increase in mIPSC frequency by 20 µM nicotine in the presence of normal extracellular calcium (Nic), pre-treatment of 0 mM extracellular calcium in the extracellular solution (Ca2+ free) and pre-treatment of 200 µM CdCl2 and 1 mM NiCl2. Comparisons among these 3 groups were made with Mann-Whitney (MW) test showing in the absence of extracellular calcium, nicotine loses its ability to increase mIPSC frequency (P = 0.0003) while the cocktail of VGCCS blockers CdCl2 (200 µM) and NiCl2 (1 mM) fails to bring down the nicotine effect (P = 0.60). (Figure used with permission).

To examine potential mechanisms of nicotine presynaptic action at GABAergic neurons, we determined whether the effect of nicotine on mIPSC frequency was calcium dependent. Calcium was removed from perfusing solution, and substituted with additional Mg2+ (Ca2+: 0 mM, Mg2+: 3.8 mM) since the nicotinic receptor can be blocked by low level of divalent ions (Adams and Nutter 1992; Liu and Berg 1999). Under these conditions, the effect of nicotine on mIPSC frequency was markedly blunted (P = 0.0003, N = 8; Figure 4.4B, D) compared to nicotine applied in normal calcium ACSF. Since our current-clamp result indicated a postsynaptic depolarizing effect of nicotine in MSNs, we examined the possible involvement of VGCCs in the enhancement of mIPSC frequency. Presumably striatal intrinsic interneurons (or other GABAergic neurons) might also be depolarized by nicotine. To completely shut down VGCC activity, we used high concentrations of CdCl2 and NiCl2 as blocking agents. Cd2+ is known to block all VGCCs, while Ni2+ is more selective for low-threshold VGCCs (Herrington and Lingle 1992). CdCl2 (200 µM) and NiCl2 (1 mM) were washed in for 10 min before nicotine (20 µM) was applied in the presence of CdCl2 and NiCl2. The results showed that despite the presence of saturating VGCC blockers nicotine was still able to induce a robust increase in GABAergic mIPSC frequency (fold increase = 9.38±1.8, P = 0.60, N = 11; Figure 4.4C, D).
4.4.4 Pharmacological characterization of presynaptic nAChR

Figure 4.5: Pharmacology of nicotinic enhancement of mIPSC frequency

A. Representative traces showing nicotine effects on mIPSCs in the presence of 1 μM mecamylamine (a) and 100 nM methyllycaconitine (b). There was no effect of 10 mM choline application (c). B. Average change in mIPSC frequency during the application of 20 μM nicotine (Nic) or 10 mM choline (Choline), and nicotine added with the antagonists mecamylamine (MEC) or methyllycaconitine (MLA). Note: in both bars the number of cells tested is indicated. Statistical significance was assessed using the MW test: nicotine (N = 15) vs. MEC (N = 4, P = 0.0191), MLA (N = 6, P = 0.40), and choline (N = 4, P = 0.0145). Asterisks denote comparison with nicotine alone group. (Figure used with permission).

The results above suggested that GABA release was enhanced following calcium influx through presynaptic nAChRs. In striatum, multiple nAChR subunits are expressed (Champtiaux et al. 2003; Wada et al. 1989; Zoli et al. 2002), including low levels of the α7 subunit (Dominguez del Toro et al. 1994; Seguela et al. 1993; Zoli et al. 2002). nAChR containing the α7 subunit are known to have a relatively high calcium permeability in
neurons (Castro and Albuquerque 1995; Seguela et al. 1993; Vernino et al. 1994) and their resulting calcium influx may cause an enhancement of neurotransmitter release (Gray et al. 1996; McGehee et al. 1995; Vijayaraghavan et al. 1992). Using different pharmacological tools, we have determined whether the nAChRs containing the highly calcium-permeable α7 subunit contribute to the stimulation of mIPSCs by nicotine. Methyllycaconitine (MLA) acts non-competitively at α7 subunit containing nAChRs and 100 nM is sufficient to block these receptors in cultured hippocampal neurons (Alkondon and Albuquerque 1993). We first co-applied nicotine with 100 nM MLA and observed no significant difference in the nicotine-stimulated mIPSC frequency (robust stimulation was still induced; P = 0.40, N = 6; Figure 4.5Ab, B). The low affinity α7 agonist 10 mM choline (Alkondon et al. 1999) failed to enhance mIPSC frequency (baseline frequency = 0.47±0.05, peak frequency during choline application = 0.78±0.10, N = 4, P = 0.17). In contrast, the relatively non-selective nAChR antagonist, 1 µM mecamylamine (MEC) was able to antagonize ~90 % of the effect of nicotine (P = 0.019, N = 4; Figure 4.5Aa, B). The lack of a α7 agonist effect (and only partial antagonist effect), but the apparent requirement for calcium entry through nAChRs (as opposed to activation of VGCCs secondary to nicotinic depolarization) suggested the involvement of other calcium-permeable nAChRs subtypes (see Discussion).

4.4.5 Effect of nicotine on spontaneous inhibitory synaptic transmission

Nicotine-stimulated mIPSCs could come from at least two populations of striatal GABAergic terminals, from fast-spiking (FS) intrinsic GABAergic interneurons and/or MSNs (Czubayko and Plenz 2002; Guzman et al. 2003; Koos and Tepper 2002; 1999; Koos et al. 2004; Tunstall et al. 2002). Striatal FS-interneurons make multiple contacts on MSNs and their eIPSCs are >4 times larger than the quantal amplitude estimated from mean-variance analysis. In the case of MSN-MSN pairs, the eIPSC and the quantal current are similar in amplitude (Koos et al. 2004). Therefore, if MSNs were to spontaneously fire, the resulting sIPSC would have the same size as the mIPSC. Assuming MSN input-derived sIPSCs are largely uniquantal we would not expect an increase in their firing rate to be associated with an increase in sIPSC amplitude. Thus we monitored nicotine-induced sIPSCs in the absence of TTX to determine whether striatal FS interneurons were excited by nicotine and formed synapses on MSNs. We determined whether the amplitude of MSN sIPSCs increased (compared to mIPSCs) reflecting
AP-mediated synchronized release of GABA from multiple FS-neuron terminals. In the absence of TTX we observed a significant increase in sIPSC frequency and amplitude during nicotine treatment (N = 3) (Figure 4.6A, B) consistent with activation of multiple release sites on intrinsic interneuron axons. To further explore the mechanism of the sIPSC changes we compared the rising phase of sIPSCs before and after nicotine application (> 20pA sIPSCs, N = 36 control and 53 nicotine; Figure 4.6Ca, b). We did not observe differences in kinetics or notches on the rising phase of single sweeps (indicating asynchronous release of quanta) (Xiang and Brown 1998), suggesting that sIPSCs reflect synaptic vesicles that were synchronously released with nicotine stimulation presumably from the stochastic firing of individual FS-interneurons and not MSNs (see Discussion).

We believe that FS-interneurons are the likely mediator of the nicotine effect based on the previous observation that interneuron-driven sIPSCs are much larger than those driven by MSN-MSN synapses (Koos et al. 2004). The observed increase in both sIPSC amplitude and frequency could only be attributed to an increase in FS-interneuron firing (which make multiple contacts with MSNs), or possibly the quantal content at each MSN or FS neuron terminal.

To further determine the nature of sIPSCs in striatum, we tested the sensitivity of nicotine-induced sIPSCs increase to 1 µM MEC, a non-selective nAChR antagonist. Similar to its effect on mIPSCs, 10 min perfusion of 1 µM MEC prior to nicotine application (in the continued presence of MEC) completely suppressed the nicotine effect on sIPSC frequency (fold increase = 1.8±0.3, N = 8, P < 0.001 as compared to nicotine alone group). The depolarization, observed during nicotine application in the presence of TTX, was also abolished by 1 µM MEC application (-75.9±0.9 mV for control before MEC perfusion, -77.2±0.2 mV for during MEC perfusion alone and -77.1±0.3 mV for during nicotine with MEC).

Since neurons were routinely held at depolarized potentials for monitoring GABAergic sIPSCs it was possible that nicotine treatment might also engage the phenomenon termed depolarization-induced suppression of inhibition (DSI) that was first observed at GABAergic synapses onto cerebellar Purkinje cells and hippocampal CA1 pyramidal neurons (Llano et al. 1991; Pitler and Alger 1992; Vincent et al. 1992). To rule out this possibility, we tested the nicotine effect at ~65 mV holding potential in neurons that were filled with an intracellular solution containing high chloride to better monitor sIPSCs at negative holding potentials. Under these conditions, robust enhancement of sIPSC frequency by nicotine was still observed at a level similar (P = 0.53, N = 4, see
supplementary figure 4.4) to the routinely applied condition (low chloride in the pipette with 0 mV holding). These results suggest that the depolarized holding potential in our experiments is not engaging forms of plasticity that modify the response to nicotine.

4.4.6 Effect of nicotine on evoked inhibitory synaptic transmission

Figure 4.6: Nicotinic modulation of evoked IPSCs

Neurons were held at 0 mV to produce an outward going IPSC mediated by GABA receptors (100 µM APV and 10 µM CNQX included). A. Nicotine enhanced the frequency
and amplitude of sIPSCs. Representative traces of sIPSCs recorded in the presence of 100 μM APV and 10μM CNQX at 0 mV holding potential and with addition of 20 μM nicotine in the bath. B. Cumulative probability histogram showing the sIPSC amplitude distribution under control conditions (255 events) and with 20μM nicotine (1026 events) from the same cell in A. Significant difference in sIPSC amplitude was observed following nicotine treatment (KS-test; P < 0.001). Ca. Representative averaged records showing sIPSC kinetics with (Nic; black trace) and without (Cont; grey trace) nicotine treatment. The ‘Nic’ trace is an average of 53 sweeps that were larger than 20 pA (amplitude). The ‘Cont’ trace is an average of 36 sweeps that were randomly chosen. Cb. Two traces in Ca are normalized to the peak current for control and nicotine cases. Note rising phase is superimposable for both cases, suggesting the large responses were not induced by accumulation of multiple quanta. D. Using a bipolar stimulus electrode placed in the striatum, IPSCs were evoked (eIPSCs) by giving 3 presynaptic stimuli at 100 ms intervals (left column) every 20 s. Addition of 20 μM nicotine resulted in a large increase in membrane noise attributed to the robust increase in sIPSC rate and an accumulation of a steady outward current presumably composed of many mIPSCs (center column). After nicotine wash out, responses were recovered partially (right column). Each trace is an average of 20 sweeps. E. Time-course of the first eIPSC in the same cell in D. During 20 μM nicotine treatment the 3 stimuli failed to produce IPSC or they were greatly attenuated in amplitude. F. Group data of the first eIPSC amplitude and sIPSC frequency plotted against time. Responses were averaged every 60 sec and normalized to an average of 5 min period before nicotine application. In the presence of 20 μM nicotine (filled circles) eIPSC was attenuated more than that in 1 μM nicotine (open circles). sIPSC frequency (stars) was enhanced by 20 μM nicotine. (Figure used with permission).

To assess the effects of nicotine-stimulated mIPSC and sIPSC activity on evoked synaptic transmission, we recorded eIPSCs from MSNs while stimulating locally within the striatum under conditions that block excitatory synaptic transmission. Using this recording configuration under control conditions, we were able to produce eIPSCs in response to presynaptic stimulation. To assess changes in release probability, we used 3 pulses of presynaptic stimulation separated by 100 ms (Figure 4.6D). In the presence of 1 μM or 20 μM nicotine (N = 7 and 6 cells respectively) we observed no consistent change in
paired-pulse ratio (2nd pulse amplitude/first, pre vs. post nicotine application; 1.22±0.76 vs. 1.58±0.60; P = 0.26 (paired t-test) for 1 μM nicotine; 0.73±0.30 vs. 1.37±0.56; P = 0.12 (paired t-test) for 20 μM nicotine see supplementary figure 4.3). Application of nicotine as in previous experiments was found to greatly elevate baseline mIPSC and sIPSC frequency (Figure 4.2, 6A). In the presence of this enhanced GABAergic activity we observed a large reduction in eIPSC amplitude (Figure 4.6D, E, F). Furthermore, there were apparent presynaptic excitation failures induced by nicotine as many trials failed to evoke synaptic response. In analyzing these data we first averaged all eIPSC traces (including failures). To better assess potential effects of increased presynaptic failure after nicotine, we also averaged responses with presumed successful stimulation during control periods, during nicotine application, and during nicotine washout (Figure 4.6E). We found that even with exclusion of failures (successful responses only), nicotine treatment resulted in a very large decrease in eIPSC amplitude, indicating a large inhibitory effect of nicotine on evoked synaptic activity. Although the depression of eIPSC amplitude partially reversed after 10 min of nicotine washout, there was a persistent increase in response failures indicating potential long-lasting presynaptic effects of nicotine. To assess the temporal relationship between nicotine effects on mini release and evoked release, we examined the frequency of sIPSCs (presumed minis and quanta released by spontaneous APs in the absence of TTX) during the baseline period preceding evoked release and observed a strong temporal relationship between depression of evoked release and enhancement of the spontaneous events (Figure 4.6F). Furthermore comparison of nicotine stimulation data from different cells under conditions that either isolate minis or enable evoked release indicate that both effects are manifested quickly within 10s of sec (see Figure 4.4A for mini time course of mIPSC elevation by nicotine). In addition to having a temporal link between high mini rates and depression of evoked release (when different cells are compared) we also find that 1 μM nicotine which produces only a small increase in mIPSC frequency also fails to depress eIPSCs, again suggesting parallels between the two processes (Figure 4.6F). Although we observed a temporal correlation between the increase in sIPSC frequency and the depression of the eIPSCs, this correlation does not mean that increases in mini frequency are sufficient to block eIPSCs. Perhaps increases in mIPSC frequency and depression of evoked activity are triggered by similar processes such as presynaptic depolarization. We speculated that eIPSC depression after nicotine application may be due to the presynaptic depolarization that leads to sodium channel inactivation.
**Figure 4.7:** Effect of low concentrations of KCl on sIPSCs and eIPSCs

Experiments were performed on striatal MSNs perfused with ACSF containing 100 μM APV and 10 μM CNQX. **A.** Membrane potentials were monitored at current-clamp mode in MSNs with 2.5, 5, 10 or 15 mM KCl. A stable membrane potential was first obtained for 10 min before altering KCl. The low KCl-containing ACSF was washed in 5-10 min until the membrane potential stabilized. **B.** Example traces showing the effect of 15 mM KCl on sIPSC and eIPSC. The stimulation scheme is the same as in figure 6. The inset shows the eIPSC before and after 15 mM KCl starts to take effect. **C.** Group data demonstrating that despite the significant depolarization caused by 10 mM KCl, a robust enhancement in sIPSC frequency does not occur until 15 mM KCl. **D.** Group data showing the eIPSCs diminishing with increasing concentration of KCl. Note: these observations were made in the absence of TTX and comparisons were made between elevated KCl group and normal.
KCl group. (Figure used with permission).

To determine the role of mild nicotinic depolarization in the mechanism of eIPSC depression we performed experiments with low concentrations of KCl in the ACSF (2.5, 5, 10 and 15 mM) to potentially mimic nicotine action. Only in the case of 15 mM KCl did we observe effects that resembled those of nicotine. 15 mM KCl was iso-osmotically substituted for NaCl in the bathing solution and the effect on eIPSC was assayed in the absence of nicotine and TTX. 5 min of 15 mM KCl application was sufficient to cause complete loss of the eIPSC (156±37 pA for control condition and 16±12 pA for during 15 mM KCl, N = 6, P < 0.001, Figure 4.7B, D). Although the eIPSC was blocked, a robust nicotine effect on sIPSC frequency was observed (fold increase = 15.4±3.4, N = 5). This result suggested that nicotinic depolarization may be sufficient to both block eIPSCs and to increase mIPSC frequency. However, we found that the magnitude of somatic depolarization elicited by 15 mM KCl (27.1±2.1 mV, N = 6, measured in current clamp, Figure 4.7A) was well above the effect of nicotine on membrane potential. To reproduce a somatic depolarization comparable to that induced by nicotine, lower concentrations of KCl were tested. As compared to the normal KCl concentration (2.5 mM), 5 mM KCl led to 7.33±0.67 mV (N = 3) of somatic depolarization (Figure 4.7A), but did not significantly reduce the eIPSC (177.8±64.1 pA to 144.8±61.5 pA, N = 8, P > 0.05) or alter mini frequency (0.66±0.19 Hz to 0.92±0.24 Hz, N = 8, P > 0.05). With 10 mM KCl, 19.2±3.2 mV of somatic depolarization (N = 3) was observed with only a marginal effect on sIPSC frequency (fold increase = 2.56±0.35, N = 6, P = 0.027, Figure 4.7C) and no significant effect on eIPSC amplitude (168±54 pA to 106±40 pA, N = 5, P = 0.38).
4.4.7 Dopaminergic modulation of striatal cholinergic enhancement of GABA activity

Figure 4.8: Effect of dopaminergic activity on mIPSC stimulation by nicotine

A Representative traces during pharmacological manipulation of nicotine-induced mIPSCs. The perfusate contains: 20 μM nicotine alone (Aa), 20 μM nicotine in the presence of 10 μM SCH23390 (Ab), 20 μM nicotine + 10 μM SCH23390 + 50 μM sulpiride (Ac), 20 μM nicotine + 50 μM sulpiride (Ad), and lastly 10 μM SKF38393 alone (Ae). Note: none of the dopaminergic drugs changed the baseline frequency of mIPSCs. B. Group data showing the effect of SCH23390, sulpiride and SKF38393 on nicotine-induced mIPSC enhancement. The reduction of nicotine (20 μM) effect by SCH23390 as shown in Ba was also observed with 100 μM nicotine for ‘Nic+SCH23390’ group and ‘Nic+SCH23390+sulpiride’ group as shown in Bb. Note: in Ba, SKF38393 application was performed in the absence of nicotine. Asterisks denote comparison with the nicotine...
alone group (the first bar). (Figure used with permission).

DA is known to modulate GABAergic transmission presynaptically in the striatum (Centonze et al. 2003; Cooper and Stanford 2001; Delgado et al. 2000a; Guzman et al. 2003). To determine whether nicotine-induced GABA release might involve dopamine, we examined the effect of D1 and D2 dopamine receptor antagonists. A combination of D1 and D2 receptor antagonists, SCH23390 (10 µM) and sulpiride (50 µM), were perfused for 10 min prior to adding 20 µM nicotine in the continued presence of the antagonists. A reduction in nicotine-stimulated mIPSC frequency was observed when compared to the nicotine alone group (fold increase = 3.8±0.7 for the dual dopamine blocker cocktail, N = 5, P < 0.05). To further determine the subtype of dopamine receptor, SCH23390 (10 µM) and sulpiride (50 µM) were tested separately. After 10 min perfusion of either SCH23390 or sulpiride, 20 µM nicotine was applied to the recording chamber. A reduction in nicotine-induced mIPSC frequency was detected when SCH23390 was administered alone (fold increase = 4.7±0.6, N = 6, P < 0.05 for comparison with nicotine alone group). In contrast, perfusion of D2 receptor antagonist sulpiride (50µM) did not alter the mIPSC frequency increase induced by nicotine (fold increase = 13.4±2.1, N = 4; P = 0.66 when compared to nicotine alone group). Therefore, the observed enhancement of GABAergic activity may in part be a result of elevated dopaminergic activity triggered by increased nicotinic activity. To more directly test this possibility the D1 receptor agonist SKF38393 (10 µM) was perfused into the recording chamber for 10 min. To our surprise, no change in mini frequency was observed (P = 0.77 when comparison was made between baseline mIPSC frequency and frequency during SKF38393 application, Fig 8Ae, Ba). These results indicate that D1 receptor activity is not directly linked to mIPSC stimulation, but may somehow be permissive for the process. A observation of the D1 receptor’s influence on nicotine-induced GABA release has been reported in substantia nigra (Kayadjian et al. 1994).

To rule out direct competition between 20 µM nicotine and DAergic antagonists, we tried the same experiment with 100 µM nicotine in the presence of 10 µM SCH23390 and 50 µM sulpiride. Compared to 20 µM nicotine, 100 µM nicotine induced a significantly larger increase in mIPSC frequency (fold increase = 27.1±5.8, N = 5, P < 0.01). Similar to data acquired with 20 µM nicotine application, perfusion of D1 antagonist SCH23390 caused a decrease in 100 µM nicotine-induced facilitation of mIPSC frequency (fold increase = 10.9±3, N = 5, P < 0.05 for comparison with 100 µM nicotine alone group).
Nevertheless, even in the presence of both D1 and D2 antagonists, SCH23390 (10 μM) and sulpiride (50 μM), 100 μM nicotine application led to a reduced enhancement of GABAergic activity as compared to 100 μM nicotine alone group (fold-increase = 10.4±3.58, N = 5, P < 0.05), confirming the data obtained with 20 μM nicotine. Overall our data suggest that D1, but not D2 receptors may impose an indirect influence on cholinergic modulation of striatal GABA activity.
4.5 Discussion

We report that nicotine can lead to a robust increase in mIPSC frequency in striatal MSNs. The robust increase in mIPSC frequency was induced by a calcium influx through calcium-permeable nAChRs and not VGCCs. Interestingly, striatal MSN mEPSCs were not enhanced in frequency or amplitude, adding selectivity to nicotinic modulation of striatal miniature activity, in contrast to lateral geniculate nucleus where nicotine enhances both types of miniature activity (Guo et al. 1998).

4.5.1 Presynaptic nAChRs enhance GABAergic transmission in a calcium dependent manner

Previous work has shown evidence for the existence of presynaptic nAChRs on GABAergic neurons. Léna and Changeux (1997) concluded that nicotine-stimulated GABA release from mouse thalamus occurs via activation of nAChRs on the nerve terminal based on the observation that nicotinic effects were TTX-insensitive. Nicotinic agonists also induce $[^3H]$-GABA release from isolated striatal synaptosomes (Behrends and ten Bruggencate 1998). Nicotine-stimulated changes in $[Ca^{2+}]_i$ were observed in synaptosomes prepared from striatum and were insensitive to VGCC blocking toxins, suggesting calcium entry through calcium-permeable nAChRs (Nayak et al. 2001). These reports are consistent with our observation that mIPSC frequency in striatum was enhanced by nicotine in a TTX-insensitive and VGCC-independent manner. Preliminary experiments using blockers of intracellular calcium stores including ryanodine, cyclothiazide, or thapsigargin had no effect on nicotine stimulated mIPSC frequency. Positive control experiments with caffeine are not feasible since caffeine has been shown to block GABAergic activity in hippocampal slices (Taketo et al. 2004), consistent with our results (data not shown). In contrast to these studies, Sharma and Vijayaraghavan (2003) demonstrated that nicotine enhanced mEPSC frequency and amplitude in CA3 pyramidal neurons resulted from calcium release from a ryanodine sensitive store following calcium influx through $\alpha 7$ type $\alpha Bgt$-sensitive nAChRs on mossy fiber terminals. Large terminals such as mossy fibers contain $\alpha Bgt$-sensitive ($\alpha 7$) nAChRs that desensitize rapidly (Couturier et al. 1990; Zhang et al. 1994). This recruitment of calcium stores would then amplify and prolong the enhanced miniature synaptic activity. In contrast, in relatively smaller terminals like GABAergic interneurons in striatum (Kubota and Kawaguchi 2000), accumulation of calcium in the terminals caused by a continuous calcium influx via non-$\alpha 7$
nAChRs could be sufficient to induce the pronounced elevation in mIPSC frequency.

4.5.2 Possible subtypes of presynaptic nAChRs

Several nAChRs subunits (α2-7, β2-4) are found in the mouse and rat striatum (Champtiaux et al. 2003; Nayak et al. 2001; Zoli et al. 2002). Although this suggests that these subunits are on presynaptic terminals within the striatum, it is not clear which cell types contain these subunits since a heterogeneous group of presynaptic terminals are also present including inputs from extrinsic DAergic and glutamatergic neurons and intrinsic cholinergic and GABAergic neurons. Champtiaux et al. (2003) and Zoli et al. (2002) showed α6 and β3 subunits of nAChRs were located on DA terminals and α4 and β2 subunits were both expressed on DA terminals and non-DA-cells or terminals. No evidence was found for α7 subunits on GABAergic neurons.

Functional nAChRs have been shown on striatal glutamate terminals in vivo (Garcia-Munoz et al. 1996) and in vitro (Kaiser and Wonnacott 2000, Wonnacott, 2000 #123). Kaiser and Wonnacott (2000) suggest that these striatal nAChRs contain α7 subunits. If sufficient numbers of α7 nAChRs are on glutamatergic terminals we should have detected an enhancement of mEPSC frequency by nicotinic agonist. However, since adult rats were used in the experiments mentioned above, it is conceivable that we did not detect nAChRs responses of α7 pharmacology since we used slices prepared from young rats. Taken together these data help to support our proposal that non-α7 subunits are likely involved in the enhancement of mIPSCs we observe. Further pharmacological analysis and/or use of knockout animals will be necessary to identify the exact subtype of nicotinic receptor involved.

4.5.3 nAChRs on GABAergic interneuron terminals trigger enhanced mIPSCs

FS-interneurons in striatum are able to induce ACh-sensitive APs (Koos and Tepper 2002) and make multiple synaptic contacts on MSNs (Koos et al. 2004). In our study we observed that nicotine application produced increases in sIPSC amplitude but not mIPSC amplitude, it is thus likely that at least a part of this effect is mediated by activation of FS-interneurons. However, there are other types of GABAergic interneurons; persistent and low-threshold spike (PLTS) neurons and calretinin-colocalized neurons are
present in the striatum (Kawaguchi 1993; Kubota and Kawaguchi 2000; Rymar et al. 2004) and could also contribute to the increase in sIPSC amplitude. Reports based on anatomical (Tepper et al. 1998) and electrophysiological studies (Koos et al. 2004) indicate that synaptic connections between MSNs (MSN to MSN) are rare in slices prepared from animals younger than postnatal day 15. Since we used animals of this age range and routinely observed increases in GABAergic transmission by nicotinic agonists we do not think that MSNs are the major source of mIPSCs. MSNs are also unlikely to be a major source of nicotine-stimulated sIPSCs since we only detected a modest subthreshold depolarization of MSNs with nicotine application (Figure 4.1Ba). Therefore, nicotine-stimulated mIPSCs recorded on MSNs could mostly come from the terminals of striatal GABAergic interneurons, like FS interneurons.

4.5.4 Possible mechanism of nicotine effect on evoked GABAergic synaptic transmission

Nicotine was found to produce a robust change in mIPSC frequency, which at times was accompanied by diminished eIPSCs (Figure 4.6). There are two possible mechanisms that might be responsible for diminished eIPSCs after nicotine enhancement of GABA mIPSCs. First, this effect might result from an occlusion of eIPSCs by depletion of transmitter following robust nicotine-stimulated mIPSCs and sIPSCs. However, assuming nicotine affects all synapses equally, it may be difficult for readily releasable pool to be depleted by nicotine stimulation of release. The nicotine-stimulated release rate at each synapse would be well below 0.01Hz, given a conservative assumption of 500 synapses on each neuron (Guzman et al. 2003; Koos et al. 2004). Since MSNs can normally follow these frequencies, we would not expect transmission to be blocked.

Another possibility is that the increased excitability and sustained depolarization of inhibitory neurons by nicotine induce inactivation of Na+ channels leading to a blockade of eIPSCs. Sustained depolarization by 15 mM KCl produced a comparable result as nicotine, i.e. enhancement of mini sIPSC frequency and profound reduction of eIPSC amplitude. In contrast, 5 mM KCl neither affected mini frequency nor altered amplitude of eIPSC, with comparable levels of depolarization as seen with nicotine application. The observation that higher levels of somatic depolarization were produced by 15 mM KCl (than nicotine) would suggest that depolarization induced sodium channel inactivation is not responsible for the nicotine-induced increase in GABA release. However, it needs to
be noted that our current-clamp recordings of nicotine-induced depolarization were performed on MSN somata instead of interneuron terminals. It is conceivable that interneuron terminals or axons may undergo stronger depolarization with nicotine leading to conduction block.

4.5.5 Indirect influence of dopamine D1 receptors on nicotine stimulation of GABA mIPSCs

Dopamine plays a critical role in striatal function as abnormal nigrostriatal projection has been implicated in diseases such as Parkinson disease both in human and in animal models (Bergman et al. 1998; Berke and Hyman 2000). In addition to targeting to major striatal projection neurons, dopaminergic inputs also terminate on striatal interneurons, where identification of receptor subtype has been complicated (Bracci et al. 2002; Centonze et al. 2003). It is possible that the observed enhancement of GABA mIPSC frequency by nicotine is mediated in part by dopaminergic neurons. To rule out an indirect effect of nicotine on mIPSC frequency through stimulation of DA release, we have performed a series of pharmacological experiments with D1 and D2 antagonists and a D1 agonist. Our results show that the D1 antagonist SCH23390 partially blocked the enhancement of GABA mIPSCs by nicotine. If dopaminergic terminals were excited by nicotine leading to dopamine release we would expect that a D1 agonist should mimic nicotine’s effect and increase mIPSC frequency. Failure of the D1 agonist SKF38393 to induce enhancement of GABA mIPSCs suggests that the action of D1 antagonists is likely an indirect effect. Our negative data with D1 agonist suggest that a direct excitatory effect of dopamine on striatal GABAergic interneurons are not sufficient to facilitate mIPSC frequency in MSNs (Aosaki et al. 1998; Bracci et al. 2002). However, we do not exclude the possibility that D1 receptors may in some way affect GABAergic mIPSCs stimulated by nicotine.

4.5.6 Physiological function of nicotine-stimulated mIPSCs

One remaining question with our study concerns the relationship between the Cl- equilibrium potential and possible depolarizing or hyperpolarizing effects of nicotine-stimulated mIPSCs. In vivo (Mercuri et al. 1991) and in acute slices the reversal potential for GABA responses was measured at ~ -60 mV with intracellular recording (Jiang and North 1991; Kita 1996; Koos and Tepper 1999) and at -64 mV with
gramicidin-perforated patch (Bracci and Panzeri 2006). In our experiments we used Cl\(^-\) concentrations that led to a -58 mV reversal potential. It is conceivable that this reversal potential may be developmentally regulated or even different for dendritic versus somatic compartments. Therefore, it is unclear whether nicotine-stimulated mIPSCs would depolarize the actual membrane potential of MSNs. The ability of nicotine to robustly increase mIPSC rates to a point where they begin to affect evoked synaptic activity suggest that nicotine could be involved in changing the mode of inhibition in MSNs from a phasic to a tonic one.

These findings raise the more general question of functional significance of minis. Perhaps scenarios exist in vivo where endogenous presynaptic modulators can increase minis to high levels. Using in vivo dialysis of TTX and intracellular recordings, Pare et al. (1997) observed that relatively intense periods of minis (~10 Hz) are common and contribute to regulation of baseline synaptic parameters such as input resistance. Assuming that a single mini is insufficient to produce AP firing by itself, conditions that promote the spatial and temporal summation of multiple synaptic inputs could more effectively modulate firing. For example, tonic miniature GABA release occurs preferentially at sites close to the AP initiation site in dentate gyrus granule cells and is likely to regulate their firing (Claiborne et al. 1986). In the CA3 region of hippocampus, mossy fibers form synapses onto the proximal dendrites of CA3 pyramidal neurons (Ishizuka et al. 1990). Given that these synaptic locations are relatively close to the AP trigger zones, nicotine-stimulated minis might easily lead to firing even in the presence of GABAergic inhibition (Sharma and Vijayaraghavan 2003). Interestingly, current injection experiments indicate that firing of electrically compact interneurons can be influenced by individual minis (Carter and Regehr 2002).

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**Supplementary figure S4.1:** Upon application of 1 μM nicotine, there is only a modest increase in mIPSC frequency (N = 4). Recordings were made in normal ACSF containing APV, CNQX and TTX, with cells voltage-clamped at 0 mV.
Supplementary figure S4.2: Effect of 0 extracellular calcium on sIPSCs.

Group data showing the effect of extracellular calcium on sIPSC frequency. sIPSC frequency in each cell was normalized to an average frequency measured over 40 sec after the addition of TTX, APV and CNQX to normal ACSF (2.5 mM calcium; open circles; N = 8) or normalized to calcium free condition (closed circles; N = 4), over the period before drugs were added in the recording chamber.
Supplementary figure S4.3: Paired-pulse ratios during nicotine suppression of evoked activity.

Paired-pulse ratio of eIPSCs were compared between before (control) and after (+ nicotine) application of 1 μM (open circles: N = 7) or 20 μM (closed circles; N = 6) nicotine. Paired-pulse ratios were calculated from 2nd/1st eIPSC amplitude ratios. Each circle shows an average value of 5 to 27 paired-pulse ratios from each cell.
Supplementary figure S4.4: Nicotine effect on GABA sIPSC is also observed at hyperpolarized potentials.

Inward sIPSCs were recorded with Cl⁻-based internal solution on striatal MSNs with membrane potential voltage-clamped at -65mV in the absence of TTX. A. Example traces demonstrating the nicotine enhancement of sIPSC frequency at -65 mV. Lower panel shows the zoomed-in activity where arrows point at ‘a’ and ‘b’ in the upper panel. B. sIPSC frequency change induced by nicotine expressed in a distribution histogram.
Supplementary figure S4.5: Suppressed nicotine effect in zero-calcium containing ACSF was rescued by substitution for bathing in the ACSF with normal concentration of calcium.

A. Example traces demonstrating the nicotine enhancement of mIPSC frequency was first suppressed in zero calcium and rescued afterwards in normal ACSF. Note that the overlap of horizontal bars denoting zero calcium and nicotine perfusion is normally sufficiently
long to induce a robust enhancement in mIPSC frequency. Lower panel shows the zoomed-in activity where arrows point at ‘a’ and ‘b’ in the upper panel. Lower traces, a, b, and c were taken from the time points indicated by arrows from the top trace. B. Frequency plot of the same trace from A.
4.6 References


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Chapter V

Discussion

5.1 Summary of Findings

In one project during my study, we have revealed a synaptic depression that lasted for about 20 min following a prolonged theta-burst stimulation protocol in hippocampal slices. A presynaptic mechanism is proposed based upon previous studies with regards to time course of postsynaptic depression and our observation with paralleled recording of AMPA and NMDA receptor-mediated currents showing a paralleled depression following the long theta-burst stimulation.

Another project dealt with a gene transcription factor, cyclic-AMP response element-binding protein (CREB). The specific aim was to establish a relationship between AP mediated calcium influx and CREB activation in primary hippocampal culture. Unfortunately, results did not prove to be conclusive due to some technical issues, although some confirmed data were obtained. For this reason, there will not be any extended discussion on this topic in this chapter. Brief discussion can be seen in Chapter 4. However, a methodological achievement was made with transfecting primary cortical and hippocampal neurons with high efficiency and good reliability in comparison with available commercial and published protocols. I recommend it to all neuroscience researchers.

In the last project described in this thesis performed with acute striatal slices, it was found that bath application of nicotine led to a robust increase in mIPSC frequency in striatal MSNs that was indicated to be dependent upon calcium influx via a calcium-permeable nAChRs and not VGCCs. Modulation of mIPSC by nicotine was reflected only in an increase in frequency but not in amplitude, suggesting a presynaptic change in vesicle release property.
5.2 Distinct Contributions of the Hippocampus and the Basal Ganglia to Learning and Memory

In this thesis, I have included three loosely related research projects that were, in reality, not attempted to relate to each other at the beginning of each of the projects. However, these three projects, synaptic plasticity, gene transcription in the hippocampus, and modulation of synaptic transmission in the basal ganglia fit well under the same category of learning and memory. In real brain, they work in concert serving dynamics of learning and memory and are discussed. Since the work on CREB phosphorylation was largely unfinished, this chapter will mainly cover findings from chapter 2 (plasticity in the hippocampus) and chapter 4 (transmission modulation in the striatum).

Broadly, the brain is not a homogenous organ: different brain areas clearly have some degree of specialized function. The hippocampus with associated neighboring cortices and the basal ganglia (BG) are thought to play distinct roles in learning and memory. Traditionally, the hippocampus has been associated with declarative memory function in humans, while the basal ganglia are associated with procedural or habit learning in animal and humans. Humans with damage to the hippocampus are often spared on a variety of tasks such as acquired learning of habits or skills. For instance, individuals with hippocampus damage along with associated cortex can learn as quickly as healthy controls in paradigms ranging from delayed eyeblink classical conditioning (Gabrieli et al. 1995; Woodruff-Pak 1993) to category learning (Maddox and Filoteo 2001). By contrast, humans with damage to the basal ganglia as in Parkinson patient often show deficits on these forms of habit learning (Sommer et al. 1999). Combining with evidence from other studies, the BG, but not the hippocampus, is proposed to play an important role in stimulus-response based habit learning (Knowlton et al. 1996; White 1997). While the hippocampus may not be critical for learning simple stimulus-response based learning, it does appear to be critical for some forms of declarative memory formation and spatial learning (Jarrard 1978; Milner 1968; Morris et al. 1982). Although the BG and the hippocampus separately execute sensory and motor tasks, they also have been shown to connect to each other functionally. Electrical stimulation delivered to nuclei in the BG has been shown to elicit theta-pattern activity in the hippocampal formation, providing support for 1), functional connection between the BG and the hippocampus, and 2), a role of the hippocampus in sensorimotor integration (Hallworth and Bland 2004).
5.3 Plasticity in the Hippocampus

5.3.1 Identification of the long train stimulation-induced depression

In this study, we have uncovered a type of synaptic depression that sustained for about 20 min following a prolonged theta-burst stimulation protocol. In conventional classification, synaptic plasticity is categorized, based upon the direction of the induced change and the length that the change lasts, into short-term depression, facilitation or potentiation (STD or STP), long-term depression or potentiation (LTD or LTP), with some special forms of plasticity such as augmentation, post-tetanic potentiation (PTP) etc (Citri and Malenka 2008). Short-term depression is, for most cases, induced by inactivation of VGCCs or depletion of presynaptic vesicles (Zucker and Regehr 2002), although the involving mechanism is still under intense investigation and debate. Either with paired-pulse protocol with paired-pulse interval shorter than 20 ms or with hypertonic sucrose as stimulant, the source of depletion normally refers to emptying synaptic vesicles from the RRP (Betz 1970; Katz and Miledi 1968; Otsu et al. 2004), where depression lasts typically from less than a second to seconds. In contrast, a homosynaptic long-term depression is typically induced by a low frequency stimulation and lasts for longer than an hour (Dudek and Bear 1992). Therefore the depression we observed does not fit into any previously established form of synaptic depression based upon its duration. In this thesis, we use long train induced-depression (LTID) to describe the depression induced by the standard 100 sec TBS protocol.

5.3.2 Studies that used prolonged high frequency stimulation in the past

At peripheral synapses such as pre-ganglionic terminal of the isolated superior cervical ganglion and neuromuscular junctions, prolonged high frequency stimulation resulted in a reduction in end-plate potential amplitude after the conditioning with a half-time of depression of 8-10 min, which is similar to our observation (Glavinovic 1995; McLachlan 1975; Naves and Van der Kloot 2001). In these studies, only a moderate reduction of the EPSPs amplitude was observed, less than 20% (McLachlan 1975). A limited number of studies have applied prolonged stimulation for driving presynaptic vesicle release in various types of synapses, with relatively consistent observation (Highstein and Bennett 1975; Krenz and Zimmermann 1978; Model et al. 1975). However, these studies have shared one thing in common, observation in large presynaptic
terminals with much larger quantal content and release probability, as compared to that in hippocampal CA3-CA1 synapses. These synapses all have intrinsic requirement for a high fidelity transmission which requires that presynaptic terminals to be equipped with release-related machineries sufficient to follow possible high frequency input. In vertebrate central nervous system (CNS), such a kind of prolonged nervous activity is not seen in thus far any physiological scenario. A prolonged high frequency discharge can only be observed brain seizure episode, which is typically followed by a suppression of electroencephalogram (EEG) activity. Unfortunately, investigation of cellular mechanisms for seizure is scarce. A recent study with intracellular recording has provided some insight of cellular aspects of seizure related neuronal activity (Nita et al. 2008). In their study, they observed an increased membrane conductance during the seizure and quick recovery after the seizure termination, along with an EPSP amplitude reduction that lasted for 5-10 min. However, no synaptic element has been probed in this study.

5.3.3 Nature of the depression

In our study, we have observed a long train induced-depression that lasted up to 27 min in hippocampal slice preparations using minimal stimulation intensity. A minimal stimulation was used to produce a mixture of success and failure of evoked EPSCs (eEPSCs). Hippocampus CA3-CA1 synapses are known to have a low release probability (Hessler et al. 1993; Rosenmund et al. 1993). Minimal stimulation has two folds of advantage in our study. One is to provide a means to better estimate the behavior of a single synapse based upon the eEPSC amplitude close to unitary EPSC or miniature EPSC (mEPSC) and its low release probability. The other advantage is that its mixture of success and failure is an effective indicator for locus of synaptic plasticity since it is generally believed that a lowered failure rate reflects an elevated release probability (Malinow and Tsien 1990). The enhanced failing of synaptic transmission to a silence level by the long train stimulation itself is a strong indication of a failure of presynaptic vesicle release. However, several alternative possibilities exist. The first is the fiber conduction failure that can be resulted from extensive stimulation to afferent fibers. One routine practice for stimulation is to use high concentration of NaCl (1 MOsm or 3 MOsm) to enhance the conductance of the electrode for more reliable stimulation. During sustained high frequency stimulation the salt content can leak out of the pipette tip and cause osmolar stress on stimulated fibers, especially for low resistance glass electrodes. In our case this is unlikely the reason since the glass pipette used for stimulation was filled with
normal ACSF that has the same electrolytic content as environmental fluid. Another important factor that can shape the synaptic transmission postsynaptically is receptor desensitization, which reflects a decreased synaptic response during sustained presence of receptor ligand. However, we think this is quite unlikely since receptor desensitization has a fast recovery kinetics and does not decline to null as in our case, even during the sustained high frequency stimulation (Arai and Lynch 1998). One comparable observation is the activity-dependent AMPA receptor endocytosis. In previous studies, it was shown that AMPA receptors can be internalized into cytoplasm or delivered to cell membrane depending the recent activity experience (Lissin et al. 1998; O’Brien et al. 1998; Turrigiano et al. 1998). In contrast to the change in AMPA receptors on the cell membrane, NMDA receptor surface expression remained unchanged. One major difference in our protocol from previous studies is that the length of the conditioning is still in the range of ‘acute’ stimulation while in contrast the conditioning used for any noticeable change in AMPA receptor expression is chronic and normally going on for days. To rule out any possible postsynaptic effect, we performed parallel recording of both AMPA and NMDA receptor mediated EPSCs and found a decrease in both AMPA and NMDA receptor mediated EPSCs. Since a change in presynaptic vesicle release is reflected in transmitter’s action on all available postsynaptic receptors, the observed reduction in both AMPA and NMDA receptor strongly suggests a presynaptic locus of the LTID. Nevertheless, when the holding potential was set to a depolarized level, LTP emerged after the depression period, indicating an unaltered postsynaptic responsiveness. However the parallel change in NMDA and AMPA receptor-mediated current can not be taken, although it does support, as an exclusive proof for a presynaptic locus of a synaptic action. A caveat is that a parallel change in AMPA and NMDA currents can also be observed in some postsynaptic modifications such as synaptic scaling, a change caused by prolonged exposure of the tissue to agents that alter basal synaptic activity (Watt et al. 2000). This is unlikely in our case since in synaptic scaling the inducing stimulation is normally a chronic treatment that lasts for hours, in contrast to our stimulation in minutes or seconds range.

One feature in our observation that is not seen in any previous study is the length of the depression induced by the long train stimulation. There are a few important features with the LTID. First, the LTID is presynaptically induced, likely a result of reduced vesicle release. This is supported by our observation on the parallel change of AMPA receptor and NMDA receptor-mediated eEPSCs and the intact LTP suggesting an unaltered postsynaptic function. Second, based upon previous studies demonstrating a relatively
fast kinetics of transmission recovery after depletion of the RRP vesicles, the long LTID period suggests a complete depletion of both the RRP and the RP vesicles. Thirdly, the differentiated synaptic response to low versus high frequency stimulation during the depression period implies a preferred replenishment to the RP compared to the RRP. Previous studies on synaptic vesicle release on showed that vesicles recycled to the RRP during a prolonged stimulation (a one minute high frequency tetanus), and vesicles recycled to the RP via infoldings and cisternae but not endosomal intermediates (de Lange et al. 2003; Heuser and Reese 1973; Richards et al. 2000). However, these studies were performed in either synapses in Calyx of Held or frog neuromuscular junctions where high transmission fidelity is required for their normal functionality. In low probability synapses like hippocampus evidence is still scarce. A recent report declared a clathrin–requirement for all modes of transmitter endocytosis with a similar rate of endocytosis from previous report from our own laboratory (Granseth et al. 2006; Otsu et al. 2004). However, conclusion from this study is difficult to reconcile with our observation on synaptic transmission after prolonged high frequency stimulation. Another elegant study on the large presynaptic terminal of the Calyx of Held demonstrated a reduced speed of reduction in cell capacitance with increasing stimulation frequency (Sun et al. 2002), suggesting slower endocytosis at high stimulation frequency. However their conclusion was based upon an untested assumption that endocytosed vesicles are exclusively made from plasmic membrane. It has been long established that in normal situation vesicles are derived predominantly from budding of the Golgi apparatus and the smooth endoplasmic reticulum in central neurons (Stelzner 1971). Therefore after a heavy synaptic drive as in our case with prolonged high frequency stimulation, it is possible that the Golgi apparatus and the endoplasmic reticulum constitutes an important supply of vesicular membrane. This speculation is supported by our observation that a short train of high frequency stimulation resumed synaptic transmission, indicating a partial refilled state of vesicle pools. This partially replenished vesicle pool can only rely on the Golgi apparatus and/or the endoplasmic reticulum for the required vesicular membrane supply, if the conclusion by Sun et al (2002) holds true in our preparation (reduced membrane retrieval as indicated by a change in cell capacitance with increasing stimulation frequency). Based upon these evidence, we proposed a hypothesized model of transmitter recycling.
5.3.4 A speculated model of transmitter recycling

A relative complete model of transmitter recycling has to provide answers to following questions.

1) What is the source of exocytosis during basal condition, experimentally during test stimulation time?
2) Where do the retrieved vesicles go back during basal condition?
3) What is the source of exocytosis during high frequency activity?
4) Where do the retrieved vesicles go back during an episode of high frequency activity?
5) Where do the retrieved vesicles go back during after an episode of high frequency activity?

In our model, we propose that the RRP sustains a basal level of synaptic activity while the RP serves to keep up the high frequency stimulation. However, this does not mean vesicles from the RRP do not release during high frequency activity. In contrast, we think vesicles from the RRP become quickly depleted during a high frequency train and release is maintained by vesicles from the RP. The size of the RRP is regulated by level of basal activity, i.e., the higher the level of basal activity the larger the RRP size; The RP size may be regulated by the strength (frequency and length of high frequency trains) the normal high frequency activity. During basal condition or test stimulation time during experiment, vesicles recycle locally in and out of the RRP while vesicles from the RP are not mobilized at this condition. During a train of high frequency synaptic activity, the RRP becomes quickly depleted and the synaptic response is sustained with mobilization of RRP vesicles to the docking site to be released. A period of high frequency activity empties the RRP to a certain degree which is important for the source of vesicle replenishment to the RP. For a short episode of high frequency synaptic input, RRP vesicles is emptied to a moderate degree and is replenished mainly via budding from the Golgi apparatus as well as from the endoplasmic reticulum. The vesicle release induced-membrane fusion results in enlargement of cell capacitance and this may trigger the formation of infolding and cisternae. The degree of vesicle recycling via infolding and cisternae is correlated with the intensity of synaptic input, i.e., the higher the afferent intensity the more infolding and cisternae are formed for vesicle recycling from the cytoplasmic membrane. With short train of high frequency stimulation, the RRP is quick
replenished from the RP where only a moderate portion of vesicles are released and are replenished quickly from membrane budding off from the Golgi apparatus and the endoplasmic reticulum. In this circumstance, no post-conditioning depression occurs. In contrast, when the synapse receives a prolonged train of high frequency stimulation, a significant portion of RP vesicles is released and the replenishment is realized via budding from large vacuoles derived from cytoplasmic infoldings and cisternae. The process how vesicles are ultimately transferred to docking site for being release ready from plasmic infoldings and cisternae remains largely an open issue to be investigated. Based upon the observation made by Richard et al (2000), it is possible that during high frequency stimulation, high level of cytosolic calcium triggers clathrin-dependent formation of endosomes and new vesicles via membrane invagination to keep up the high frequency activity, although newly formed vesicles have diluted level of transmitter. In the meantime, the cytoplasmic infolding and cisterna may form but do not participate in vesicle recycling. The formation of these large vacuoles may represent a transient form of membrane carrier to be transported back to the Golgi apparatus and the endoplasmic reticulum. Vesicle formation via this route may represent a calcium-independent recycling mechanism (Koenig and Ikeda 1996). After the high frequency episode, the cytoplasmic infoldings and cisternae bud off from the plasmic membrane and form large vacuoles that are then transported back to the core of synaptic terminals to fuse with either the Golgi apparatus or the endoplasmic reticulum. From there, new vesicles are pinched off from these cytoplasmic organelles and undergo reacidification of vesicular fluid and gradual transmitter reuptake into the inside of the vesicle. What can be directly suggested from our observation is that after complete depletion of vesicles from entire presynaptic pools, vesicles in the RP is preferentially replenished prior to be translocated to the RRP. Alternatively speaking, newly formed vesicles after a high frequency stimulation will not be transported to the docking site if not sufficiently refilled with transmitter molecules. In our experiments, about 20 min is required for the vesicles in the RP to be refilled and translocated from the RP to docking site after vesicles from both the RRP and the RP are depleted.
At basal condition (before LTS), a small number of vesicles are sitting on a release site forming the readily-releasable pool (RRP). A much larger number of vesicles are held back by microtubules and actins forming the reserve pool (RP). A prolonged train stimulation is capable of depleting vesicles from the entire presynaptic terminal. Immediately after the stimulation, there is no effective vesicles formed other than some clathrin-coated empty vesicles and membrane invaginations. Shortly after the LTS, the invaginations, also called membranous infoldings or cisternae, start to leave plasmic membrane and form some large vacuoles that can bud off vesicles. Some vesicles, possibly the large vacuoles as well, start to have reuptaken transmitter in the content. At this time, the RP is mixed with vesicles that are empty, partially filled or completely filled. But no vesicle is primed for release at this time. After a sufficiently long time, 20 min in our case, all the vesicles are refilled with transmitter and some of them are recruited to the RRP to be release ready. Note that in reality the vesicles from the RP and the RRP may not be as clearly segregated as in our schematic plot.

Conventionally, presynaptic vesicles are divided into three different pools, RP, RRP and a recycling pool (Rizzoli and Betz 2005). The recycling pool is defined as vesicles that cycle in the vicinity of release site in response to moderate stimulation or short burst of high frequency stimulation (de Lange et al. 2003; Harata et al. 2001; Richards et al. 2000). Evidence has shown that vesicles from the RP and the RRP are not spatially segregated clearly (Harata et al. 2001; Micheva and Smith 2005). From our observation with prolonged stimulation protocol, all vesicles from the RRP as well as the RP participate in effective transmission depending the stimulation frequency and length of the stimulation. The previously defined recycling pool vesicles include all vesicles from the RRP and a small portion of the RP since the studies where the original definition derived from engaged stimulation with short train of stimulation. The size of the recycling pool has
never been clearly declared. Combining previous studies and my observation it is likely that there is no independent recycling pool. Previously defined recycling pool is no more than a portion of vesicles from the RP mobilized by high frequency stimulation. However, there is a tempting possibility that vesicles from the RP that are geographically close to release sites have less restraint from actin and microtubules based upon the moderately faster kinetics of release of initial period after depletion of vesicles from the RRP (Neves and Lagnado 1999). This speculation is yet to be investigated.

One pending question is what the rate limiting process is that determines the length of the synaptic depression after a prolonged high frequency input that depletes both vesicle pools. For a typical transmitter cycling in the CNS, the transmitter is first released to synaptic cleft, dissipating to extrasynaptic interstitial matrix, then gets reuptaken by the presynaptic terminal or transported into adjacent glial cells. Upon strong synaptic input when the presynaptic terminal is partially deprived of its vesicles, the transmitter molecule will generally be produced in the presynaptic terminal or transported from adjacent glial cells to the terminal. However, glutamate cycling has a special mechanism distinct from other transmitters. Released glutamate is reuptaken by Na⁺-dependent excitatory amino acid transporter EAAT-1 and EAAT-2 that expressed predominantly on astrocyte surface, with minimal direct reuptake into presynaptic terminals (Chaudhry et al. 1995; Rothstein et al. 1994). Recent work has demonstrated expression of an alternatively spliced EAAT2 isoform at presynaptic terminals in the hippocampus (Chen et al. 2004), but its physiological role remains uncertain. In neurons utilizing glutamate as transmitter, glutamate is derived from precursor glutamine (Hamberger et al. 1979b). Intrasynaptic glutamate must be kept low in order to maximize the signal-to-noise ratio after the release. This is accomplished by rapid uptake of glutamate into astrocytes, which convert glutamate into glutamine by glutamine synthetase (Pow and Crook 1996; Pow and Robinson 1994; Rothstein and Tabakoff 1984; Winkler et al. 1999). Upon some physiological requests from neurons, glutamine can then transferred first out of astrocytes via system N transporter and then into neurons via system A transporters (Broer 2008; Chaudhry et al. 1999). This process has been conventionally named as glutamate-glutamine cycle. It has been shown that retardation of glutamine supply by inhibiting the biochemical process impaired the aversive learning in chick, suggesting an important role of non-neuronal supply of glutamine in the chick CNS (Hertz et al. 1996). Including glutamine in perfusing solution can prevent presynaptic vesicle depletion induced by strong stimulation (Storm-Mathisen et al. 1986), precluding the rate limiting
role of glutamine being uptaken into neurons and converted to glutamate in neurons. Therefore, it might be of special interest to see how fast the stored glutamine can be converted into glutamate and get released. Although seemingly not a difficult task, there has been no evidence showing the rate of astrocyte supply of glutamine upon heavy synaptic inputs. In normal physiological circumstances, the RP, as indicated by its name, serves as a reserve reservoir of synaptic vesicles to cope with some heavy release demand upon severe scenarios. At least in human, there is no circumstance other than in a life-threatening epilepsy breakout that would deplete these RP vesicles. Based upon the rapid conversion of glutamine to usable glutamate in neurons (Storm-Mathisen et al. 1986), a likely rate limiting process after depletion of both RRP and RP vesicles by prolonged heavy synaptic drive resides in the glutamine synthesis reaction chain. The kinetics of the glutamine synthesis reaction chain in astrocytes is yet to be determined.

5.3.5 Future direction:

5.3.5.1 Examination of calcium dependence of the endocytosis during the depression period following the prolonged high frequency stimulation

To explore mechanisms underlying the observed synaptic depression following a prolonged high frequency stimulation, one important question we can not ignore is the calcium-dependency of vesicle replenishment during the depression. It has been shown that both exocytosis and endocytosis is dependent upon calcium entry via distinct routes (Kuromi et al. 2004). Calcium source for endocytosis is less understood compared to that for exocytosis. Vesicle refilling after heavy synaptic activity has been shown to be independent of extracellular calcium change (Ramaswami et al. 1994). A recent study demonstrated that the RP refilling via endocytosis induced by high potassium depended upon the calcium released from internal stores (Kuromi and Kidokoro 2002). Upon heavy synaptic drive, released glutamate can spill out of the synaptic cleft and activate a variety extrasynaptic receptors located on perisynaptic regions (Liu et al. 1998). One of the important receptors located on presynaptic terminal that can be activated by heavy synaptic stimulation are mGluRs. mGluRs are known to be coupled to adenylate cyclase that can hydrolyze phosphoinositide (PI) and liberate diacylglycerol, which activates PKC and inositol 1,4,5-triphosphate (IP3), which causes release of Ca^{2+} from intracellular stores (Pin and Duvoisin 1995). mGluRs can be categorized into three groups. Group I is mainly localized on postsynaptic site while certain members of group II and III
are predominantly located on presynaptic regions (Anwyl 1999). Although the dominating function of mGluRs is to suppress synaptic transmission with their negative coupling to adenylate cyclase and phospholipase C (Schoepp 2001), a more recent study have revealed that activation of presynaptic mGluR group I induced an increase in intracellular calcium possibly from internal stores (Schwartz and Alford 2000). It is possible that the prolonged high frequency stimulation applied in our study likely causes glutamate spill-over to activate presynaptic mGluRs that in turn can lead to a sustained activation of presynaptic internal calcium stores. With pharmacological agent that can deplete internal calcium stores, such as thapsigargin, it is possible to exhaust the calcium content from internal stores prior to the conditioning stimulation. If the internal store indeed participates in endocytosis of transmitter back into the RP, a lengthened period of depression is expected to occur. However endocytosis is complicated by many factors. The involvement of internal store supply of calcium can alternatively be manifested in reduced capacity of transmitter release, i.e. compromised ability to follow high frequency stimulation and possible faster recovery of normal transmission.

5.3.5.2.: Possible modification of the synaptic depression induced by prolonged high frequency stimulation by application of glutamate precursor.

Immunocytochemistry study has shown that in hippocampal slices addition of glutamine in perfusing solution can prevent presynaptic terminals from depletion of presynaptic glutamate immunoreactivity, suggesting an enhanced endocytosis with addition of glutamate precursor (Storm-Mathisen et al. 1986). In related discussion of the glutamate-glutamine cycle, we have mentioned that glutamate supply for transmission is derived from perisynaptic astrocytes. Based on these facts, we can investigate whether glutamine can serve similar function in our preparation. It is predicted that with addition of glutamine in our perfusates the depression period induced by the 100 sec theta burst stimulation will become greatly shortened. In contrast, a glial-specific metabolic blocker, for instance fluoroacetate, would exert an inhibitory effect on glutamine supply from astrocytes and hence make the long synaptic depression from prolonged high frequency stimulation even longer.
5.3.5.3 Optical resolution of the synaptic depression induced by prolonged high frequency stimulation

Generally speaking, electrophysiology recording does not provide direct support per se for phenomena related to transmitter release. Recent advances in using capacitance measurement as an index for transmitter vesicle endocytosis and exocytosis have provided much insight in understanding the mechanism of synaptic vesicle cycling (von Gersdorff and Matthews 1999). However, it is flawed by its inability to distinguish actions of different synaptic vesicle pools. By using prolonged high frequency stimulation in patch-clamp recording of synaptic currents, this issue was solved, partially. However, our conclusion remains highly speculative. The ultimate solution resides in optical probing into presynaptic terminals for changes in those vesicles. Here we provide an experiment design that may address the issue using optical measurement.

A styrylpyridinium molecule, FM1-43 can be used due to the fact that this molecules fluoresce at acidification with its lipophillic tail stuck in the vesicular membrane and its positively charged heavy head preventing it from leaking through the membrane. The experiment involves a parallel measurement of presynaptic terminal staining with optical imaging and simultaneous recording of postsynaptic currents with patch-clamp electrode. The easiest preparation is autaptic culture since the same electrode will provide both stimulation signal and current recording. After a loading stimulation (2Hz for 10 min, effective loading protocol will be optimized with different combinations), we expect a relatively complete loading of both RP and RRP vesicles. Then the residual dye in the extracellular solution will be washed off. Then the typical stimulation protocol in our electrophysiology experiment (100 sec theta burst stimulation) will be delivered to destain the presynaptic terminal. Based upon our electrophysiology observation, the presynaptic terminal fluorescence should be completely vanished at this time. Shortly (about 1min) after the conditioning stimulation, FM1-43 will be washed in again in an attempt to reload the presynaptic terminals and be washed out at the time point right before an attempt is made to monitor the terminal staining. Based upon the conclusion derived from our electrophysiology experiments, we expect to detect the FM1-43 staining during the depressed period indicated by EPSC recording, which would suggest a loading preferentially into the RP. One important parameter we can obtain with this approach is that the time required for a complete depletion of presynaptic vesicles can be determined with varying the length of stimulation protocol. We can even replicate the experiment in our electrophysiology recording with the 20 Hz train (20 pulses) in an attempt to destain the
RP vesicles to a certain degree. This would consolidate our preliminary conclusion that high frequency stimulation primarily drives release of vesicles from the RP. However, primary culture by far does not represent the physiology in acute slices as in the current study, let alone living tissue. Therefore, any result obtained from a compromised preparation should be interpreted with sufficient caution.

5.3.6 Conclusion

We have observed an about 20 min long synaptic depression derived from a prolonged theta burst stimulation (100 sec). It suggests that a longer period of time is required for replenishment of vesicle in the RRP if vesicles from the RP are also depleted, compared to replenishment induced by depletion of only RRP vesicles. Combining with a supporting experiment and other’s finding (de Lange et al. 2003; Harata et al. 2001; Richards et al. 2000), it is also suggested that the vesicle replenishment preferentially goes to the RP compared to the RRP replenishment. With the establishment of the time required for complete depletion of both RRP and RP vesicles, one can effectively observe the interaction of the RP and the RRP and develop a better understanding of mechanisms that underlie the synaptic transmission under both physiological and pathological situations. Our finding also provides a cellular analogy for clinically observed depression in electroencephalography (EEG) after seizure episodes.
5.4 Modulation of Synaptic Transmission in the Basal Ganglia

5.4.1 Mechanisms of enhancement of GABAergic transmission by nAChRs

In acute striatal slices, we have observed a robust enhancement of GABAergic activity by extracellular perfusion of nicotine. This enhancement of GABAergic minis by nicotine application was calcium-dependent as indicated in the abolished effect after perfusing with zero-calcium-containing solution. However, the nicotinic effect on GABAergic minis was not affected by application of a non-selective VGCC blocker, suggesting that calcium-permeable nicotinic receptors were in part mediating the effect. Nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels composed of five subunits surrounding a water-filled, cation-permeable pore (McGehee and Role 1995; Role and Berg 1996). nAChRs have three classifications, muscle nAChRs found in the mammalian muscular junctions and two neuronal types. One neuronal type of receptors takes on a form of hetero-oligomeric combination with $\alpha$ and $\beta$ subunits ($\alpha$2-6, $\alpha$10 and $\beta$2-4) whilst the other type has a homo-oligomeric composition with only one $\alpha$ subunit ($\alpha$7-9) (Albuquerque et al. 1997; Dani 2001). The combination of $\alpha$4 and $\beta$2 represents the most common form of nicotinic receptors that is widely expressed in the central nervous system (CNS). The next common form of nicotinic receptor type is the type made of homo-oligomeric $\alpha$7 subunits. Of the subunits capable of forming homomers, only the $\alpha$7 subunit is widely distributed in the mammalian brain. One important feature for $\alpha$7 nAChRs is their high calcium permeability compared to other types of nicotinic receptors with predominant localization on presynaptic terminals (Fabian-Fine et al. 2001; Seguela et al. 1993; Zarei et al. 1999). This suggests that the calcium-dependence of the nicotinic effect on GABAergic mini enhancement may be mediated via these calcium-permeable nicotinic receptors. Our results indicated, in contrast to our expectations, that the effect was not blocked by the $\alpha$7 homomer specific antagonist despite the good sensitivity to non-selective nicotinic antagonism. This suggests that the observed effect of nicotine on GABAergic activity is mediated a non-$\alpha$7 containing calcium-permeable nicotinic receptor.

Within the striatum, cholinergic neurons represent one of three major classifications of interneuron, cholinergic large aspiny neuron, also known as tonically active neurons (TANs), the parvalbumin/GABAergic medium aspiny neuron (Kemp and Powell 1971; Kita and Kitai 1988), and the somatostatin, neuropeptide Y, and NADPH diaphorase.
containing aspiny neurons (Gerfen and Wilson 1996). Among these three major type of interneurons, cholinergic neurons plays a central role in modulatory operations in the striatum. They are normally large in size and account for 1~2% of the striatal neuronal population (Bolam et al. 1984; Phelps et al. 1985). Striatal cholinergic interneurons, by definition ‘interneurons’, serve modulatory action in local circuitries, although a subpopulation of them also projects to the neocortex (Parent 1990). These accounts suggest that the enhancement of GABAergic activity by extracellular perfusion of nicotine mimics a local effect of cholinergic interneurons. In the striatum, cholinergic interneurons make reciprocal innervations on GABAergic neurons (Limberger et al. 1986; Raiteri et al. 1990). Therefore, the effect of nicotine upon GABAergic MSNs can be either direct or indirect. If nicotine acts upon MSNs directly on postsynaptic nicotinic receptors, the postsynaptic nature of action can be reflected by a change in IPSC amplitude as well as frequency. However, our results indicated that only frequency but not amplitude of mIPSC responded to nicotine application with a robust increase, suggesting that the action of nicotine is not directly on postsynaptic nAChRs but likely in an indirect manner on a presynaptic terminal or somata of an inhibitory neurons. In the striatum, over 90% of striatal neurons are MSNs. However, local inputs onto MSNs are predominantly from interneurons instead of among MSNs themselves, especially for rats younger than day 15 as in our case (Koos et al. 2004). However, occasional connections between MSNs were reported for reasons yet to be unraveled (Taverna et al. 2004). Previous studies also suggested that GABAergic interneurons were preferentially activated with field electrical stimulation within the striatum (Jaeger et al. 1994; Kita 1996; Koos and Tepper 1999; Tepper et al. 1998). This indicates that GABAergic interneurons are likely to be the primary candidate for receiving endogenous cholinergic inputs.

Previous studies have shown that primary synaptic targets of the parvalbumin-containing fast-spiking (FS) interneurons are MSNs (Bennett and Bolam 1994; Tepper et al. 2004). These FS interneurons form local circuits with MSNs and impose the most influential actions on MSNs. APs in FS neurons induce large unitary inhibitory postsynaptic potentials (IPSPs) and bursts of APs can depolarize postsynaptic MSN membrane potential by up to 7 mV (Koos and Tepper 1999; Koos et al. 2004). These observations are consistent with our results in that nicotine application increased only the amplitude of sIPSCs, but not mIPSCs, suggesting that FS interneurons constitute the major synaptic target of nicotinic modulation from cholinergic neurons in our experiments. However, other types of GABAergic interneurons may also mediate nicotinic effects on
MSNs. The ultimate proof relies on the challenging experiments in which electrophysiological recordings of both the innervating GABAergic interneuron and the receiving MSN are performed. Although nAChRs have diverse functions in the CNS, one of the key modulatory functions is their modulation via presynaptic terminals to enhance release of a wide variety of neurotransmitters (Gray et al. 1996; McGehee et al. 1995). As for cholinergic innervation on FS neurons, immunocytochemical evidence showed that both somata and terminals possessed synaptic connections with cholinergic interneurons (Chang and Kita 1992). In our results, the nicotinic enhancement of GABAergic activity was dependent upon calcium. A calcium transient initiated from a remote region in the neuron normally relies on a calcium signal relay by calcium stores (Hardingham et al. 2001). This was not the case for our study where calcium store blockers had no influence on the nicotine-induced GABAergic mini increase, suggesting that presynaptic terminals instead of somata of FS interneurons are likely the target for the extrinsic action by nicotine. In addition, the limited space in presynaptic terminals of GABAergic interneurons is beneficial for sufficient elevation of calcium via the presynaptic calcium-permeable nAChRs which impose a change in release probability. Evidence suggest that these calcium-permeable nicotinic receptors can initiate sufficient calcium influx to evoke transmitter release (Gray et al. 1996; Lena and Changeux 1997). Taken together this suggests that the observed nicotinic effect on GABAergic mini enhancement represents a scenario in which cholinergic neurons exert a presynaptic modulation of GABA release from the FS interneuron terminal to postsynaptic MSNs.

One important finding from our study is the dampened eIPSC during the enhancement of GABAergic activity of MSNs by nicotine application. Miniature activity represents synaptic current recorded from transmission without explosion of an AP in postsynaptic neurons (AP-independent) while the evoked synaptic response denotes the summated postsynaptic response from many APs (AP-dependent). Depending upon the number of inputs and concurrent release probability, the evoked response can be transformed into an AP firing. Our data present a scenario where miniature activity can affect effective synaptic transmission that may result in AP firing depending upon concurrent modulatory factors. The proposed mediating interneuron is the parvalbumin-containing FS GABAergic interneuron that is characterized with a high frequency bursting upon a large depolarization. However, these interneurons are quiet at rest and rarely fire spontaneous APs, at least in slice preparations (Koos and Tepper 2002), consistent with our observations for the basal mIPSC frequency range (0~1.5 Hz).
(Chapter 4, Figure 4.2). This leaves a wide space for an up-regulated mini activity to reduce the probability of effective inhibition conveyed by APs, releasing the inhibition on MSNs and thereby enhancing the inhibitory output from the striatum. Nonetheless, evidence from other studies have shown that the corticostriatal excitatory LTD induced by high frequency electrical stimulation is dependent upon modulation from dopamine release (Partridge et al. 2002). Therefore, as dopaminergic modulation, nicotinic enhancement of GABAergic activity may represent a unconventional plasticity by providing a modulatory tone, which may be generalized to other brain regions.

5.4.2 Interaction between dopaminergic and cholinergic receptors

Our data showed that the effect of nicotinic receptor activation upon GABAergic activity can be influenced by dopaminergic D1 receptors but not by D2 receptors. It carries a potential implication for future therapeutic strategy for degenerative diseases such as Parkinson’s disease. Understanding basal ganglia physiology has been greatly benefited from performing researches in Parkinson’s disease therapy. Most pioneering works have proposed a simple model regarding how cholinergic and dopaminergic effects interact in the striatum. There are two counteracting pathways working in concert controlling the motor function, the direct pathway and the indirect pathway. The net effect of the direct pathway is excitatory with the presence of two GABAergic synaptic connections in the circuitry, while the net effect of the indirect pathway is inhibitory with three synaptic connections in the circuitry. At rest, the net output for the thalamus is inhibitory, indicating a dominating role of the indirect pathway in the motor control (Albin et al. 1989). The early view for interactions between the dopaminergic input and the cholinergic modulation is as such. The cholinergic system in the striatum exerts an excitatory net effect on striatal output to the thalamus, i.e., an enhanced inhibitory net effect upon the motor tone. The dopaminergic innervation was originally thought to impose a balanced control on the motor function with the cholinergic system in the striatum, by inhibiting ACh release from cholinergic terminals. Therefore a loss in dopaminergic control on ACh action results in a motor suppression as reflected in hypokinesia seen in Parkinson’s disease. This model has been widely accepted partly due to the successful amelioration of symptoms of Parkinson’s disease patients with use of L-Dopa. The later discovery of the dopamine receptor subtypes, D1 and D2, has improved the model, stating that dopaminergic input imposes an excitatory influence on the direct pathway through D1 receptor activation in contrast to an inhibitory effect on the indirect pathway through D2 receptor activation.
(Sealfon and Olanow 2000). However, this is still an oversimplified model with many complications. For example, although D1 and D2 receptors appear to be largely segregated, there is a substantial subpopulation (~20–25%) of MSNs that coexpress these receptors (Surmeier et al. 1998). The situation was further complicated by the discovery of more dopaminergic receptor subtypes, such as D3 and D4. D4 is expressed at low levels in the striatum (Bergson et al. 1995) while D3 is expressed in 40% of neurons in the direct pathway (Bordet et al. 1997). Direct evidence showing interaction between dopaminergic and cholinergic actions has been poorly advanced (Lehmann and Langer 1983; Sandor et al. 1991). In our study, we showed that the enhancement of GABAergic mini activity by presynaptic nicotinic receptor activation was diminished by the dopaminergic D1 receptor but not by the D2 receptor antagonist. The inhibitory effect upon the cholinergic system in the striatum by the dopaminergic system in the classical model was found to be mediated by D2 receptor in the indirect pathway (Maurice et al. 2004; Salgado et al. 2005). In the classical model describing the mechanisms on cholinergic system interaction with dopaminergic system in the striatum, muscarinic receptor activation has been thought to be responsible for the cholinergic excitation on the motor control. And D2 receptor activation is now thought to be responsible for inhibition of cholinergic function by suppressing acetylcholine release (Maurice et al. 2004; Salgado et al. 2005). The mAChR and the D2 receptor perhaps represent the critical elements influencing the striatal output. Interestingly, cholinergic activity can also maintain the AP-dependent dopamine release by nicotinic receptor activation. Blockade of nicotinic receptor or depletion of acetylcholine vesicles similarly reduced dopamine release by ~90%, presenting an intriguing contrast to mAChRs (Zhou et al. 2001). It is provoking a challenging possibility that while mAChRs counteract with dopaminergic D2 receptors in motor control, nAChRs may be playing a modulatory role by maintaining dopamine release. Our data suggest that the D1 receptor may play a similar role in cholinergic function in the striatum. The failure of the D1 receptor agonist on the nicotinic effect on GABAergic activity, combining with the positive effect of the D1 antagonist, is reminiscent of the permissive role of D-1 receptor stimulation by endogenous dopamine on rodent yawning behavior where the antagonist SCH23390 reduced yawning while the agonist SKF38393 failed to induce yawning (Longoni et al. 1987). It raises the possibility that D1 receptor activity may be permissive for nicotinic action on GABAergic interneurons. Strong supporting evidence also comes from a previous study on a high frequency stimulation-induced corticostriatal synaptic long-term depression (LTD) (Partridge et al. 2000).
Dopamine by itself did not induce any change in synaptic efficacy. The observed LTD was dependent upon an AP and dopamine release that was sustained by nAChR activation, as well as supporting the above-proposed modulatory role of nAChRs in dopamine release. Based mainly upon a previous study and our observations, the model may be modified as such. mAChRs impose an inhibitory function to the final motor control by enhancing the inhibitory output to the thalamus and the cortex. Dopamine, released from dopaminergic terminals extended from cell bodies in substantia nigra par compacta, acts on D2 receptors inhibiting ACh release in the striatum. D1 receptor activation, in contrast, plays a permissive role in cholinergic function. nAChRs, once activated, contribute to modulation of dopaminergic function by maintaining dopamine release.

5.4.3 Future experiments:

5.4.3.1 Identifying interneurons that mediate the nicotinic effect upon MSN GABAergic mini enhancement

Based upon previous experiences and our observations, it is preliminarily proposed that parvalbumin-containing FS GABAergic interneurons respond to presynaptic nicotinic modulation, resulting in an enhancement of GABAergic activity in MSNs. A challenging but feasible experiment can be performed which involves dual recordings of a MSN and a GABAergic interneuron. To confirm the recorded interneuron belongs to the FS type, the interneuron is recorded with a current-clamp mode while the MSN is recorded with a voltage-clamp mode. The effective connection of the two recorded neurons can be confirmed with an evoked sIPSC in the MSN by eliciting APs in the recorded interneuron. A series of current steps can be delivered to the supposed interneuron to identify its cell type. A typical FS interneuron should respond to a depolarizing current injection with a high frequency AP train. If depolarization is sustained, bursts of AP train should be seen with a relatively regular interval (Koos and Tepper 2002). If identified as a FS GABAergic interneuron, a similar procedure can be applied to test the response of the recorded MSN to the extrinsic application of nicotine while monitoring the membrane potential change of the FS interneuron. In response to nicotine application, the FS interneuron should respond with a depolarization, the level of which would depend upon the dose of nicotine applied. A sufficient level of depolarization should drive the interneurons to fire high frequency APs. A similar firing pattern invoked by nicotine application and current injection into the interneuron could occur in the recorded MSN.
Similar control experiments have to be done with non-FS interneurons that have synaptic contacts with the MSN. The mini increase invoked by current injection into the recorded non-FS interneuron should not be seen with nicotine application, confirming the mediation of nicotine activity via the FS GABAergic interneuron.

5.4.3.2 Examination of graded modulatory tone imposed by nAChR activation

Our results demonstrated that 20 μM nicotine application induced a robust enhancement of GABAergic activity in MSNs mediated by presynaptic nicotinic receptors on GABAergic interneurons. It provides a modulatory tone for inhibitory synaptic transmission. However, it may only represent an extreme scenario where only modulatory effect from very strong nicotinic influence can be seen. It is possible that different levels of nicotinic tone may have distinct influences upon GABAergic modulation of MSN minis. At rest, cholinergic neurons fire tonically at a relatively steady frequency, providing a basal tone for up- and down-regulation of GABAergic activity. For example, the cholinergic tonic tone can respond with a pause to a motor task (Aosaki et al. 1995). These changes are ultimately reflected in the GABAergic activity they modulate. With a similar experimental scheme as described in the Chapter 4, a graded concentration of nicotine can be tested for its presynaptic modulation of GABA release. Distinct responses are predicted. At high concentrations as seen with 20 μM in Chapter 4, the evoked response may suffer from vesicle depletion due to a robust increase in GABA release. In contrast, at low concentrations, nicotine may play a role restricted to mobilizing the vesicle release from the GABAergic interneuron, thereby enhancing the evoked IPSC.
5.5 Summary

In this thesis, two scenarios are presented in the hippocampus and the striatum stating that prolonged high frequency stimulation of presynaptic machineries lead to depletion of synaptic vesicles, with distinct physiological consequences in different brain regions. In the hippocampus, the prolonged high frequency stimulation caused an arrest or depression of synaptic transmission due to the proposed depletion of the entire presynaptic vesicle pool, before the transmission gradually climbed back to a new plateau level in about 20 min. In the striatum, the prolonged stimulation with nicotine application dampened the evoked IPSC, possibly as a consequence of a partial depletion of vesicles of the RRP. The complete depletion of the entire presynaptic vesicle pool as possibly occurred in the hippocampus induced by the prolonged high frequency stimulation in our study, in our opinion, may represent a universal model that provides the over-stimulated neuron protection from further responses to possible pathogenesis stimulation. In contrast, caution should be taken for attempts that tend to generalize the conclusion drawn from our study with the striatum. In the case of the striatum, the physiological consequence varies depending upon the type of interneurons as reported in the hippocampus (Ji and Dani 2000), the level of enhancement induced by varying concentration of nicotine applied. In this regard, the take-home message would be that a depletion of the entire presynaptic vesicle pool with both the RRP and the RP may serve fundamentally different purpose from a depletion of only the RRP vesicles.
5.5 References


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Appendix

A.1 Detailed method for transfection of hippocampal neurons with calcium phosphate

Transfection of primary cultured neurons has been experimentally challenging in the past mainly due to general cell health compromise after transfection. In this study, we have modified a transfection protocol based upon a previously reported method of transfection (Kohrmann et al. 1999) and developed a customized method of transfecting primary cultured neurons with calcium phosphate. The detailed description is as below.

Transfection solution:
1. HBSS washing buffer (pH 7.4): 135 mM NaCl, 20 mM HEPES, 4mM KCl, 1mM Na₂HPO₄, 2mM CaCl₂, 1mM MgCl₂, 10 mM glucose.
2. Transfection buffer (pH7.1 sharp) or 2xBES-buffered saline (2xBBS): 50mM BES, 1.5mM Na₂HPO₄, 280mM NaCl.
3. NMEM-B27 transfection medium (pH7.5): MEM (Gibco: Cat11700-077; Lot1166084), Sodium pyruvate 1mM; NaHEPES 15 mM, L-glutamine 2 mM (Invitrogen Canada Inc.), D-glucose 33 mM, B-27 (50ml for 500 ml MEM); pH7.5, 305 mOsm adjusted with D-mannitol (Sigma-Aldrich).

Transfection procedure:
1) Warm up NMEM-B27 (NMEM+) medium in the dish for transfection in 37°C, 0% CO₂ incubator.
2) By the time the medium is warmed (10 min is sufficient), transfer coverslips into petridishes. Let neurons sit for half an hour in the transfection incubator (37°C, 0% CO₂). At this time, take out the transfection reagents out of fridge to warm them up in the room temperature.
3) During the waiting, start to prepare DNA plasmid for transfection. For 10 cm dish, take 15 μg plasmid into 1.5 ml tube. Put a tenth volume 3 M sodium acetate of DNA solution. Mix. Put 3x volume 100% ethanol of DNA solution. Tap the bottom of the tube to mix the solution and one will see the appearance of DNA pellet. Then add 1 ml 100% ethanol. Spin the tube in 4°C room for 2 min at 14,000 RPM. By the end of spinning, immediately pour out the ethanol from the tube. The pellet should stay visible at the bottom. Once the DNA pellet is dried, put 400 ul 250 mM CaCl₂ into the aliquot to dissolve the DNA pellet. (Now the cell dish should be ready in the hood) Put 400 ul 2x BBS solution into
the DNA solution. Gently pipette up and down to homogenize. Immediately after this, put the transfection mix drop-wise into the cell dish. (By this time, the experimenter should make sure that no bubble is underneath coverslips so that swirling does not move coverslips around. If bubble found underneath, press the coverslip in the middle using the tip of curved forceps.) Swirl the dish to homogenize the plasmid-containing medium (watch the coverslips for possible floating due to tiny bubbles underneath). Put the cell dish into the transfection incubator (37°C, 0% CO₂) and let it stay until a layer of find crystals appears as an indication for terminating the transfection (typically about 2 hours).

For dishes with different sizes, follow the dose below:

<table>
<thead>
<tr>
<th>Dish size</th>
<th>3 cm</th>
<th>6 cm</th>
<th>10 cm</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA amount</td>
<td>4 ug</td>
<td>8 ug</td>
<td>15 ug</td>
</tr>
<tr>
<td>medium volume</td>
<td>2 ml</td>
<td>5 ml</td>
<td>10 ml</td>
</tr>
<tr>
<td>Transfection mix</td>
<td>160 ul</td>
<td>400 ul</td>
<td>800 ul</td>
</tr>
</tbody>
</table>

4) Fine CaPO₄ crystals form typically between 1~2 hrs. wash the cells with pre-warmed HBSS for 3 times with cells sitting in the cell dish (no bubbles underneath). Put cells back to 24 well plate containing normal neural basal medium and send back to normal incubator (37°C, 5% CO₂) for desired period for sufficient expression of interest proteins. (Taking transfection day as day 0, peak expression for GFP is day 3)

A.2 Comment on transfection method

Neurons incorporated with gene introduced with this transfection method normally yield high efficiency and good reliability without noticeable compromise of general health, likely resulting in neurons good for a wide variety of experiments including high quality-demanding electrophysiology experiments. Although the yield is generally higher and more reliable than conventional transfection methods from commercial kits, there is variability associated with a number of factors. One is the health of cultured neurons. There is a normal variation in neuron’s health condition between cells from different batches of culturing, or different dishes cultured from the same culture batch. Compromised neuron health is, from our observation, associated with a reduced level of transfection efficiency. Although compromised, the transfection efficiency is still no less
than 5%. Another is the age of cultured neurons. Young neurons normally yield significantly higher transfection efficiency than older neurons. However, there is a window that this consideration applies. In neurons younger than 3 DIV, cells may be too fragile to take on the calcium phosphate challenge. For older neurons, neurons normally start to die after 30 DIV even with good maintenance in incubators. In our case, neurons older than 25 DIV normally do not survive the transfection and remaining neurons yield very low transfection efficiency, less than 10 neurons on the 12 mm coverslip. Third factor is cell density. From our experience, if plating density is lower than 20,000 cells/ml, there tends to be many single neurons with no neurite contact with other neurons. These isolated neurons are much less resilient to shock provoked by calcium phosphate and suffer from more cell death after calcium phosphate treatment. From our experience, 100,000 cells/ml is preferred for the highest transfection efficiency and reliability, with denser plating toward compromised transfection efficiency and sparser plating toward cell sickening. Lastly, the size of precipitate crystals matters. In general, higher transfection efficiency is associated with larger calcium phosphate crystals and vice versa.

### A.3 Keys for a successful transfection

There are only two major factors critically determining the fate of transfection. One is the size of crystal particles. Crystals with good size normally form a fine-sand looking layer of particles that are almost transparent in color. Elevated toxicity is expected if crystals turns opaque due to their larger size. On the opposite, if the crystal particles are too small even to be detected, normally no transfected neurons would be seen afterwards. The key for an appropriately sized particles resides in the pH of the medium after 2xBBS is added into the NMEM+ medium. Therefore, pH of 2xBBS and NMEM+ should be precisely made and maintained. In our practice, 2x BBS and NMEM+ were made on the same day after pH meter was calibrated twice. Then both 2xBBS and NMEM+ are aliquoted for each experiment with caps sealed with parafilm and afterwards stored at 4°C. Such solution aliquots gave us consistent transfection up to at least six months.

In my opinion, this is the key for transfecting primary cultured neurons in contrast to cell line transfection such as human-embryonic kidney cells (HEK cells). Traditionally the difficulty of transfecting primary cultured neurons comes from the toxicity imposed by large calcium phosphate particles if protocols for cell lines are used for neurons. It is possibly because cell lines differ in their membrane property from primary cultured neurons in a way that makes them more resistant to extrinsic toxicity. Calcium phosphate crystals of
reduced size carry the gene of interest into the cytoplasm and have no significant toxicity to the affected neuron, therefore making transfection of primary cultured neurons an easier task for many of our researchers.

The other important factor is transfection duration. A transfection with appropriately sized crystal particles does not guarantee a successful outcome with routine procedure. The transfection efficiency and toxicity is also affected by the time during which neurons are treated with transfection buffer (2xBBS). In our practice, aging neurons tend to more resilient to calcium phosphate-induced toxicity and have lower transfection efficiency with the same transfection time as young neurons. Therefore it is recommended to lengthen the transfection time to a certain period of time based upon experimenter’s observation for those older cultures. In our practice, we kept our transfected neurons for half an hour longer in the transfection incubator (0% CO₂) upon crystal formation before the transfection medium is washed and replaced. For neurons younger than 10 DIV, we terminate the transfection immediately upon crystal formation.

A.4 Speculated mechanism of transfection of primary cultured neurons with classical calcium phosphate method

The mechanism below is proposed based upon my observation and is currently in a highly speculative state, therefore requires further experimenting.

The size of precipitated crystals in calcium phosphate treated wells varies significantly due to even slight variation of acidity in medium incubating neurons. Under microscopes, calcium phosphate crystals exhibit peculiar physical phenomenon, autonomic vibration, unless calcium phosphate is added so overdosed as to form super large crystals or even aggregates of these crystals. For a number of times in my experience, those calcium phosphate crystals were mistaken as bacteria or fungi contamination due to their vibrating appearance, by a number of my colleagues. The inorganic nature of these vibrating particles was confirmed by growing a small portion of the medium containing vibrating particles in a new petrodish for days without any change in medium color indicating that there is no organism growing in the medium. This peculiar vibration forms the key for my proposal as follow: calcium phosphate crystals formed in the moderately acidic media environment may represent an energetically unstable state that endows crystals the vibrating profile. The vibrating crystals wiggle their way randomly in
the neuron growing dish and can penetrate the cell membrane and become stuck on the membrane. Stuck crystal particles ruptures the membrane, each to a small extent, and allows free ion exchanges between inside and outside of the neuron, causing depolarization and subsequent toxicity, to a varying degree depending upon the crystal size with larger crystals leading to more damage of the membrane. At the mean time, the plasmid carrying genes of interest attach to the crystals and utilize them as vehicle to be transferred into the neurons. Once the plasmid carrying the gene of interest is released into the cytoplasm, plasmids finish their journey and start to produce the protein of interest.
A.5 References