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Karina Leal

Fine-tuning Synaptic Plasticity by Modulation
of Presynaptic $Ca_V2.1$ Channels with Ca^{2+} Sensor Proteins

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Abstract

Fine-tuning Synaptic Plasticity with Calcium Sensor Proteins

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Short-term plasticity of synaptic transmission is recognized as an important component of information processing in neuronal networks. Repetitive firing in neurons either leads to an increase, or to a decrease in synaptic transmission, processes referred to as facilitation and depression. This short-term plasticity behavior of synaptic transmission is specifically regulated at each given synaptic connection. P/Q-type and N-type Ca^{2+} currents through $Ca_v2.1$ and $Ca_v2.2$ channels, respectively, are responsible for the Ca^{2+} entry that initiates neurotransmitter release at most conventional synapses. However, it remains not well understood to what extent regulation of $Ca_v2.1$ channels play a role in short-term plasticity. Recent work has shown Ca^{2+}-dependent regulation of $Ca_v2.1$ channels to be mediated by calmodulin (CaM) and neuronal Ca sensors (CaS), which include calcium binding protein 1 (CaBP1) and visinin-like protein 2 (VILIP-2). Mutations of the CaS binding sites in the carboxyl terminal of $Ca_v2.1$ affect short-term synaptic plasticity. Although it is clear that CaS-dependent regulation of $Ca_v2.1$ channels induces synaptic plasticity, it remains unknown which CaS proteins are responsible for these changes.

Candidate proteins include, CaM, CaBP1, and VILIP-2, which show differential modulation of $Ca_v2.1$ channels in heterologous expression systems; yet, their role in synaptic transmission has not been studied. Here, we show that activity-dependent modulation of presynaptic $Ca_V2.1$ channels by CaBP1 and VILIP-2 has opposing effects on short-term synaptic plasticity in superior cervical ganglion (SCG) neurons. Expression of CaBP1, which blocks Ca^{2+}-dependent facilitation of P/Q-type Ca^{2+} current, markedly reduced facilitation of synaptic transmission. VILIP-2, which blocks Ca^{2+}-dependent inactivation of P/Q-type Ca^{2+} current, reduced synaptic depression and increased facilitation under conditions of high release probability. These results demonstrate that activity-dependent regulation of presynaptic $Ca_V2.1$ channels by differentially expressed CaS proteins can fine-tune synaptic responses to trains of action potentials and thereby contribute to the diversity of short-term synaptic plasticity.

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GLOSSARY

Ca^{2+}	calcium
Ca_V	voltage-gated Ca^{2+} channel
EGTA	ethylene glycol-bis (beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid
RRP	readily releasable pool
CaS	Ca^{2+} sensor
I_{Ca}	Ca^{2+} current
MNTB	medial nucleus trapezoid body
CNS	central nervous system
VGCC	voltage-gated Ca^{2+} channel
CDF	Ca^{2+}-dependent facilitation
CDI	Ca^{2+}-dependent facilitation
CaM	calmodulin
IM	IQ-like motif
CBD	calmodulin binding domain
CaBP1	calcium binding protein 1
VILIP-2	visinin-like protein-2
VILIP-3	visinin-like protein-3
VILIP-1	visinin-like protein-1
NCS-1	neuronal calcium sensor 1
SCG	superior cervical ganglion
AP	action potential
EPSP	excitatory postsynaptic potential
PPR	paired-pulse ratio
ISI	inter-stimulus interval
PPF	paired-pulse facilitation
PPD	paired-pulse depression
STP	short-term plasticity
tsA-201	non-neuronal cell line
eGFP	enhanced green fluorescent protein
FGD	fast-green dye

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Dedication

This thesis is dedicated to my parents, Norma Y. Leal and the late Juan Leal, who always supported me throughout my educational pursuits, taught me never to give up, and who in turn never gave up on me.

Chapter I

Introduction and Background

Synaptic transmission involves a highly complex series of events by which electrical potential across the membrane of presynaptic nerve terminals regulates the release of neurotransmitter release. Ca^{2+} ions play a critical role in this process: when an action potential invades a presynaptic terminal, Ca^{2+} influx through voltage-gated Ca^{2+} (Ca_V) channels leads to a rise in intracellular Ca^{2+} concentration, followed by binding of Ca^{2+} to a presynaptic Ca^{2+} sensor that triggers synaptic vesicle fusion, thereby releasing neurotransmitters into the synaptic cleft. The released neurotransmitters diffuse into the synaptic cleft, bind to postsynaptic receptors, and ion channels open, which leads to a change in membrane potential of the postsynaptic cell. In this way, the signal is transferred from one neuron to another. Synaptic transmission is not a static process. Postsynaptic responses strengthen and weaken as presynaptic activity changes, and this form of plasticity is crucial for neuronal network processing (Zucker and Regehr, 2002).

Short-Term Synaptic Plasticity

The strength of synaptic transmission can vary during repetitive presynaptic activity and depends heavily on the previous activity of a synapse. Neurons fire at frequencies ranging from less than once per second (1 Hz) to several hundred Hz at specialized synapses in the brain (von Gersdorff and Borst, 2002). Short-term plasticity represents an increase or decrease of synaptic strength, which lasts from hundreds of milliseconds to seconds during repetitive stimulation (Abbott and Regehr, 2004; Zucker and Regehr, 2002). The same

stimulation pattern does not necessarily induce the same form of synaptic plasticity at different synapses (Ding et al., 2008; Dittman et al., 2000). All synapses show some form of short-term facilitation, depression or a combination of both facilitation and depression. The input-output relationship between the pre- and postsynaptic neurons can be dynamically modulated by short-term synaptic plasticity, determining how the signal is transformed from one neuron to the other (Zucker and Regehr, 2002). However, the cellular and molecular mechanisms underlying short-term plasticity are not well understood.

For many decades, researchers have been trying to understand the mechanisms that underlie changes in transmitter release. Short-term plasticity is predominantly presynaptic although postsynaptic mechanisms have been identified and well characterized, like postsynaptic receptor desensitization and saturation (Del Castillo and Katz, 1954b; Zucker and Regehr, 2002). Short-term plasticity can result in synaptic enhancement through three processes- facilitation, augmentation, and post-tetanic potentiation (PTP)- all of which vary in time of onset and duration (Zucker and Regehr, 2002). Short-term plasticity may also lead to a decrease in neurotransmitter release, resulting in synaptic depression (Zucker and Regehr, 2002). Here we will discuss the background for synaptic facilitation and depression, the study of augmentation and PTP are beyond the scope of this study. Many presynaptic mechanisms have been identified that potentially play a role in either synaptic facilitation and depression; but one common factor is that they are both Ca^{2+}-dependent (Xu et al., 2007). For facilitation, the “residual Ca^{2+} hypothesis” is the most prevalent hypothesis. Residual Ca^{2+} refers to Ca^{2+} remaining in the presynaptic terminal upon action potential (AP) invasion of the synaptic terminal. The level of residual Ca^{2+} is sub-micromolar and decays slowly following depolarization (Katz and Miledi, 1968). If a second AP comes a short time later,

elevation of local $[Ca^{2+}]_i$ through voltage-gated calcium channels (VGCC) is added to the remaining residual Ca^{2+}. It is postulated that more Ca^{2+} is available to bind to the Ca^{2+} sensor for neurotransmitter release and induce a larger postsynaptic response to the second AP. Thus, presynaptic Ca^{2+} entry during the first AP is critical for facilitation (Del Castillo and Katz, 1954a; Dudel and Kuffler, 1961; Katz and Miledi, 1968). This implies that Ca^{2+} influx is responsible for facilitation, at least, in some conditions. This hypothesis was confirmed by the observation that presynaptic introduction of a slow Ca^{2+} buffer, ethylene glycol-bis (2-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), which prevents this increase in Ca^{2+} concentration, diminished facilitation (Atluri and Regehr, 1996; Cuttle et al., 1998; Habets and Borst, 2005). The increase in the size of readily releasable pool (RRP) and the Ca^{2+} sensitivity for vesicle fusion do not contribute to facilitation of transmitter release (Felmy et al., 2003; Hori and Takahashi, 2009), but are relevant for potentiation of release in response to activation of second messengers (Kaneko and Takahashi, 2004; Sakaba and Neher, 2001). However, it remains unknown how residual Ca^{2+} leads to various forms of synaptic facilitation. The simplest and most prevalent hypothesis is that residual Ca^{2+} binds to the Ca^{2+} sensor for neurotransmitter release and therefore increases synaptic vesicle release (Katz and Miledi, 1968). On the other hand, recent data suggest that residual Ca^{2+} enhances release probability by binding to a Ca^{2+} sensor different from the putative Ca^{2+} sensor for evoked neurotransmitter release (Sippy et al., 2003; Tsujimoto et al., 2002; Zucker and Regehr, 2002; Atluri and Regehr, 1996). Thus, the effector mechanism through which residual Ca^{2+} enhances neurotransmitter release is unknown.

Short-term synaptic depression, observed during trains of stimuli or when the second of two closely timed stimuli is depressed compared to the first, has been proposed to result from

depletion of vesicles from the RRP at synapses with high initial release probability (Zucker and Regehr, 2002). Classical studies in which depletion of the total pool of synaptic vesicles was observed directly by electron microscopy required trains of action potentials that lasted 10 to 30 minutes. However, for rapid synaptic depression in paired pulse or short trains this hypothesis is still under debate and poorly understood (Sullivan, 2007; Xu and Wu, 2005). A decrease in the release probability downstream of the presynaptic Ca^{2+} current (I_{Ca}) and a decrease in presynaptic I_{Ca} itself have both been shown to contribute to synaptic depression (Forsythe et al., 1998; Wu and Borst, 1999; Xu and Wu, 2005). Thus, multiple processes may be involved in the process of synaptic depression, but the molecular mechanisms remain poorly understood.

Role of Ca^{2+} Channels in Short-Term Synaptic Plasticity

Although these two hypotheses, residual Ca^{2+} and depletion of the RRP, have been studied extensively, a wave of recent studies at the calyx of Held synapse have shown that short-term plasticity is also achieved by a mechanism involving regulation of presynaptic $Ca_V2.1$ channels, even though their contribution had been considered minimal previously (von Gersdorff and Borst, 2002). The calyx of Held is a large specialized mammalian central synapse in the medial nucleus of the trapezoid body (MNTB). Because of its large presynaptic terminal, it is possible to record both presynaptic Ca^{2+} currents and postsynaptic responses simultaneously (Eggermann et al. 2011; von Gersdorff and Borst, 2002).

In the central nervous system (CNS), P/Q-type Ca^{2+} currents conducted by $Ca_V2.1$ channels are the major sources of Ca^{2+} entry into presynaptic compartment (Dunlap et al., 1995). $Ca_v2.1$ channels are localized in high density in nerve terminals where they initiate neurotransmission at fast conventional synapses in the CNS (Catterall, 2000; Westenbroek et

al., 1995). Several studies at different synapses has shown neurotransmitter release to be proportional to the presynaptic Ca^{2+} current or the presynaptic Ca^{2+} concentration raised to the third or fourth power, so small changes in Ca^{2+} entry have large effects on synaptic transmission (Augustine et al., 1987; Dodge and Rahamimoff, 1967b; Zucker and Regehr, 2002). Thus, modulation of presynaptic Ca^{2+} channels that lead to changes in Ca^{2+} influx provides an efficient way to modulate neurotransmitter release.

$Ca_v2.1$ channels are composed of an $\alpha1$ subunit and auxiliary subunits β, $\alpha_2\delta$, and sometimes γ (Catterall, 2000). The auxiliary subunits significantly affect the function of the channel (Dolphin, 2003; Hofmann et al., 1999). The $\alpha1$ subunit is the largest subunit and is composed of four homologous domains (I-IV), each consisting of six transmembrane α-helices (S1-S6). The $\alpha1$ subunit forms the pore of the channel and contains the voltage sensor unit (S1-S4) and pore module (S5-S6) (Figure 1.1*B*). The large intracellular loops of the $\alpha1$ subunit, including the N- and C-termini, serve as sites of interaction by different regulatory proteins that influence Ca^{2+} signals and channel function (Catterall, 2000; Catterall and Few, 2008).

Consistent with the "residual Ca^{2+} hypothesis", Ca^{2+}-dependent facilitation of presynaptic calcium currents by residual Ca^{2+} also contribute to facilitation of transmitter release at synapses (Borst and Sakmann, 1998a; Cuttle et al., 1998). Recent data have shown $Ca_V2.1$ channels, but not $Ca_V2.2$ channels, undergo activity- dependent facilitation (Borst and Sakmann, 1998a; Cuttle et al., 1998; Forsythe et al., 1998; Tsujimoto et al., 2002). When external Ca^{2+} is replaced with Ba^{2+}, facilitation of $Ca_v2.1$ channels is reduced suggesting that Ca^{2+} mediates this effect (Cuttle et al., 1998). At the calyx of Held, Ca^{2+}-dependent facilitation of presynaptic I_{Ca} has been shown to correlate with a component of short-term

facilitation (Inchauspe et al., 2004). In $Ca_v2.1$ knock-out animals, facilitation of the presynaptic Ca^{2+} current and postsynaptic responses are absent (Ishikawa et al., 2005). This study suggests Ca^{2+} entry through $Ca_V2.1$ channels specifically results in Ca^{2+}-dependent facilitation of I_{Ca} and synaptic facilitation. Moreover, inactivation of the $Ca_v2.1$ channels correlates with rapid depression at the calyx of Held synapse. Only during high stimulation conditions (100Hz) was depletion of the RRP the dominant mechanism for depression (Xu and Wu, 2005).

Further, a recent study using recombinant $Ca_V2.1$ channels expressed in cultured superior cervical ganglion (SCG) neurons demonstrated that the Ca^{2+}-dependent facilitation (CDF) and Ca^{2+}-dependent inactivation (CDI) of $Ca_V2.1$ channels are mediated through neuronal CaS proteins that bind the $Ca_V2.1$ channel carboxyl terminus and induce short-term facilitation and rapid synaptic depression, respectively (Mochida et al., 2008). The $Ca_V2.1$ channels are upstream of neurotransmitter release; due to the power relationship between Ca^{2+} influx and transmitter release, CDF and CDI of these channels may efficiently alter synaptic efficacy.

Ca^{2+}/CaS-dependent Regulation of $Ca_v2.1$ Channels

Ca^{2+}-dependent facilitation and inactivation of presynaptic Ca^{2+} channels are observed at the calyx of Held synapse in the rat brainstem (Forsythe et al., 1998; Inchauspe et al., 2004). During activity-dependent stimulation at this synapse, $Ca_v2.1$ channel currents show both Ca^{2+}-dependent facilitation and inactivation, which contribute to facilitation and depression of excitatory postsynaptic (Borst and Sakmann, 1998b; Cuttle et al., 1998; Forsythe et al., 1998).

Ca^{2+}-dependent facilitation and inactivation are also observed for cloned and expressed $Ca_v2.1$ channels expressed in mammalian cells (Lee et al., 2000; Lee et al., 1999). The mechanism for Ca^{2+}-dependent facilitation (CDF) and Ca^{2+}-dependent inactivation (CDI) of $Ca_v2.1$ channels as tested in non-neuronal cells involves Ca^{2+} sensor protein calmodulin (CaM) binding to two adjacent sites on the C-terminus—the calmodulin binding domain (CBD) and the upstream IQ-like motif (IM) (DeMaria et al., 2001; Lee et al., 1999; Lee et al., 2003). The IM site is required for facilitation, whereas the CBD is required for inactivation. Mutation of the IQ-like domain, IM-AA, completely prevents CDF of $Ca_v2.1$ channels, whereas deletion of the CBD inhibits CDI of $Ca_v2.1$ channels (Lee et al., 1999; Lee et al., 2003). Similarly, regulation of $Ca_v2.1$ channels by CaM is lobe-specific. Mutation of the two EF hands in the carboxyl-terminal lobe primarily prevents facilitation, whereas mutation of the EF hands in the amino-terminal lobe primarily prevents inactivation (DeMaria et al., 2001; Erickson et al., 2001; Lee et al., 2003). A molecular model has emerged based on these experiments that describe Ca^{2+}/CaS-dependent facilitation and inactivation of $Ca_v2.1$ channels by CaM. When $Ca_v2.1$ channels open, a local rise in intracellular Ca^{2+} activates the two high-affinity C-terminal Ca^{2+} binding EF-hands of CaM, which initiates or strengthens an interaction with the IQ-like motif causing facilitation. Following prolonged Ca^{2+} entry, an increase in global intracellular Ca^{2+} leads to binding of Ca^{2+} to the lower-affinity N-terminal EF-hands of CaM, where it interacts with both the IQ-like domain and CBD to produce inactivation (DeMaria et al., 2001; Lee et al., 1999; Lee et al., 2003). This biphasic regulation of $Ca_v2.1$ channels by CaM may be the process by which neurons undergo CDF and CDI (Xu et al., 2007; Mochida et al., 2008; Catterall and Few, 2008). In neurons, both Ca^{2+}-dependent facilitation and inactivation are blocked by

coexpression of a CaM inhibitor peptide (Lee et al., 1999; Xu and Wu, 2005), suggesting that Ca^{2+}-dependent modulation of $Ca_v2.1$ channels in neurons is caused by two sequential interactions with CaM or a related Ca^{2+}-sensing protein. $Ca_V2.2$ and $Ca_V2.3$ channels have CDI that depends on CaM, but neither shows evidence of Ca^{2+}/CaS-dependent facilitation of Ca^{2+} channel activity (Liang et al., 2003). These data suggest Cav2.1 channels are unique in that regulatory CaS proteins may modulate CDF where these channels are specifically expressed.

Neuronal Calcium Sensor Proteins: CaBP1 and VILIP-2

The broad range of diverse Ca^{2+} induced phenomena in neurons, ranging from neurotransmission to gene expression (Berridge et al., 2003), is reflected by the discovery of a large number of different Ca^{2+} sensing proteins (Burgoyne, 2007; Burgoyne et al., 2004). CaM is the founding member of a large family of related Ca^{2+} sensor (CaS) proteins that are primarily expressed in neurons in the brain and retina (Braunewell and Gundelfinger, 1999; Burgoyne and Weiss, 2001; Haeseleer et al., 2002; Haeseleer and Palczewski, 2002). CaS proteins transduce the Ca^{2+} signal to other cellular components; bound Ca^{2+} induces a conformational change resulting in the exposure of hydrophobic surface(s), which induces new interactions with target proteins (Burgoyne and Weiss, 2001). Regulation of the function of the target proteins results in a wide range of physiological changes that lead to signaling specificity generated by the existence of multiple Ca^{2+} sensors that have different properties and specific target proteins (Burgoyne, 2007; Burgoyne et al., 2004; Burgoyne and Weiss, 2001). CaS proteins share similar structural motifs to CaM (Figure 1.2*B*). CaM has four functional EF-hand motifs, N-terminal EF hands (1 and 2) and C-terminal hands (3 and 4) that respond to the coordination of Ca^{2+}, joined by central α-helix (Burgoyne and Weiss,

2001; DeMaria et al., 2001; Lee et al., 2003). More than 40 members have already been identified in the neuronal CaS family (Burgoyne and Weiss, 2001). The members of the neuronal CaS family possess a large range of sequence homology, ranging between 37 and 100% identity at the amino acid level (Burgoyne and Weiss, 2001). Like CaM, all CaS proteins contain 4 putative EF-hand motifs although the first or second EF-hand is often not able to bind Ca^{2+} (Burgoyne, 2007). Many share a consensus sequence for N-terminal myristoylation (Burgoyne, 2007; Burgoyne and Weiss, 2001; Haeseleer et al., 2002; Mikhaylova et al., 2011). Moreover, Ca^{2+} affinities ranging from low nM to low μM concentrations are well poised to bind physiological Ca^{2+} at a faster rate than CaM (O'Callaghan and Burgoyne, 2003). Included in this superfamily are the neuronal CaS proteins calcium binding protein 1 (CaBP1) and visinin-like protein 2 (VILIP-2) that are similar in structure to CaM but unlike CaM are localized primarily in neurons (Haeseleer et al., 2000; Paterlini et al., 2000). These neuronal CaS are thought to be implicated in the fine modulation of neuronal sensitivity and excitability (Burgoyne et al., 2001). Previous research has established neuronal CaS proteins in the brain and retina as essential modulators of voltage-gated Ca^{2+} channels in heterologous expression systems (Lautermilch et al., 2005; Tsujimoto et al., 2002; Lee et al., 2002). Their exact *in vivo* function remains generally elusive. CaBP1 is expressed in the cerebral cortex, hippocampus, and in the retina (Haeseleer et al., 2000). VILIP-2 is mainly expressed in the brain (cortex, hippocampus, hypothalamus, midbrain, olfactory bulb) except the cerebellum and pons (Saitoh et al., 1994; Paterlini et al., 2000). The highest amount of VILIP-2 mRNA is observed in the hippocampus. Only a small amount is detected in the cerebellum (Paterlini et al., 2000; Kajimoto et al., 1993). Like CaM, CaBP1 and VILIP-2 bind to the CaM-binding sites in the C-terminus of $Ca_v2.1$

channels (Lautermilch et al., 2005; Lee et al., 2002). CaBP1 binds to the CBD of $Ca_v2.1$ channels, but with regulatory effects different from those of CaM. In tsA-201 cells, CaBP1 causes rapid inactivation of $Ca_v2.1$ channels in a Ca^{2+}-independent manner. During trains of depolarization, CaBP1 causes enhanced inactivation and prevents CDF compared to endogenous CaM (Figure 1.2*A* and Figure 1.2*C*; Lee et al., 2002; Few et al., 2011). In contrast, both the IQ-like motif and the CBD are required for VILIP-2 to mediate its effects. VILIP-2 causes slow inactivation in a Ca^{2+}-independent manner, and enhances facilitation and prevents CDI (Figure 1.2*A* and Figure 1.2*C*; Nanou et al., 2012; Lautermilch et al., 2005). This differential effect by CaM, CaBP1, and VILIP-2 in regulating $Ca_v2.1$ channels is a potentially important determinant of Ca^{2+} entry in neurotransmission. How modulation of $Ca_v2.1$ channels by CaM and neuronal CaS proteins contribute to regulation of synaptic transmission and short-term plasticity remains unknown.

CaS proteins and Short-term Synaptic Plasticity

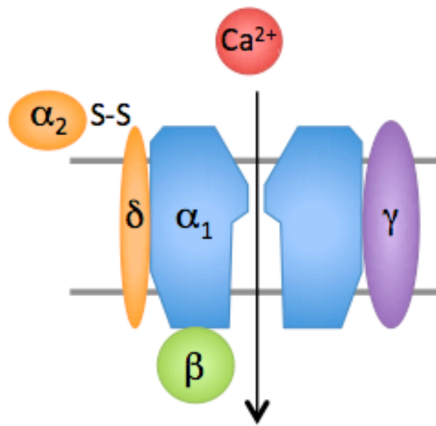
Recent studies suggest residual Ca^{2+} in the active zones of presynaptic terminals may act on CaS proteins (Tsujimoto et al., 2002; Sippy et al., 2003; Mochida et al., 2008). Application of neuronal calcium sensor-1 (NCS-1) increases presynaptic $Ca_v2.1$ channel current by accelerating the activation of the channel (Tsujimoto et al., 2002). In cultured hippocampal neurons, overexpression of NCS-1 similarly enhances synaptic transmission (Sippy et al., 2003). These results suggest residual Ca^{2+} facilitates $Ca_v2.1$ channels by binding NCS-1. Mutation of CaS binding sites on the C-terminus of $Ca_V2.1$ channels prevents CaS binding and consequently prevents synaptic facilitation and depression (Mochida et al., 2008). It is possible that differential expression of the many neuronal CaS proteins add to the diversity of synaptic behavior during repetitive activity. Because

regulation of $Ca_V2.1$ channels by CaBP1 and VILIP-2 is strikingly different from CaM, it would be predicted that differential expression of different neuronal CaS proteins in different synapses would result in different ratios of synaptic facilitation and depression.

Although it is clear that CaS-dependent regulation of $Ca_v2.1$ channels induces synaptic plasticity (Mochida et al., 2008; Tsujimoto et al., 2002), it remains unknown which CaS proteins are responsible for these changes and how they alter channel function. Candidate CaS proteins include, CaM, CaBP1, and VILIP-2, which show strikingly different modulation of $Ca_v2.1$ channels in heterologous expression systems (Figure 1.2*C*); yet, how this different regulation of $Ca_V2.1$ channels will be reflected in synaptic transmission has not been studied. Therefore, the purpose of this thesis study is to examine the regulation of presynaptic $Ca_v2.1$ by CaS proteins in short-term synaptic plasticity.

Figure 1.1 Structural aspects of voltage-gated Ca^{2+} channels.

(A) The subunit composition of $Ca_V2.1$ channels: pore forming $\alpha1$ subunit, and auxiliary subunits β, $\alpha2\delta$, and γ. **(B)** Putative transmembrane topology of the $\alpha1$ subunit. The $\alpha1$ subunit is composed of four homologous domains (I-IV) each with six transmembrane segments (S1-S6). The IQ-like domain (IM, red), and the CaM-binding domain (CBD, purple) are sites of interaction for calmodulin (CaM) and neuronal calcium sensor (CaS) proteins. The sites of interaction of different regulatory proteins on the intracellular surface of the $\alpha1$ subunit of Ca_V2 channels are also illustrated.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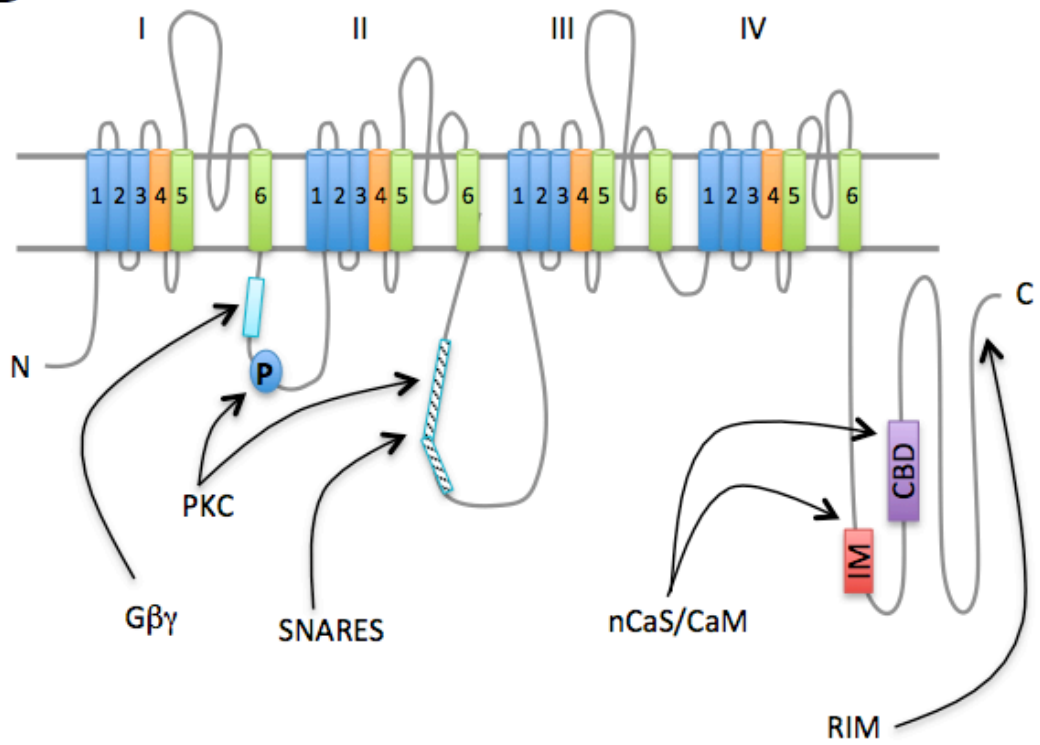
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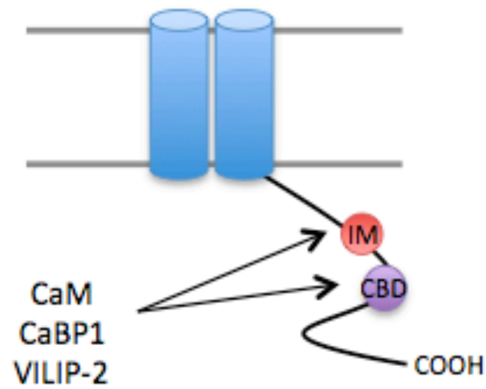
SNARES
CBD
IM
nCaS/CaM
RIM

Figure 1.2 Regulation of $Ca_V2.1$ channels by CaM and neuronal CaS proteins.

(A) Key structural domains within the $Ca_V2.1$ channel c-terminus involved in its interaction with the neuronal CaS proteins calmodulin (CaM), Ca^{2+}-binding protein 1 (CaBP1), and visinin-like protein 2 (VILIP-2). CaM and VILIP-2 bind to both the IM site (red) and CBD domain (purple), whereas the CBD domain is the site of action of CaBP1. Mutation or deletion of both carboxyl sites abolishes regulation by these neuronal CaS proteins. **(B)** Schematic representation of CaM, CaBP1, and VILIP-2, showing N-terminal myristoyl group (CaBP1 and VILIP-2), EF-hands, and central α-helical linker. Filled boxes represent functional EF-hands for binding Ca^{2+}. Open boxes denotes inactive EF-hands unable to bind Ca^{2+}. **(C)** Averaged normalized Ca^{2+} current (I_{Ca}) amplitudes elicited by repetitive depolarizations for 5 ms to 20 mV at 100Hz in tsA-201 cells expressing $Ca_V2.1$ channels modulated by endogenous CaM (black) or overexpressed CaBP1 (blue) and VILIP-2 (green) (**B-C**, Adapted from Catterall and Few, 2008).

A



B

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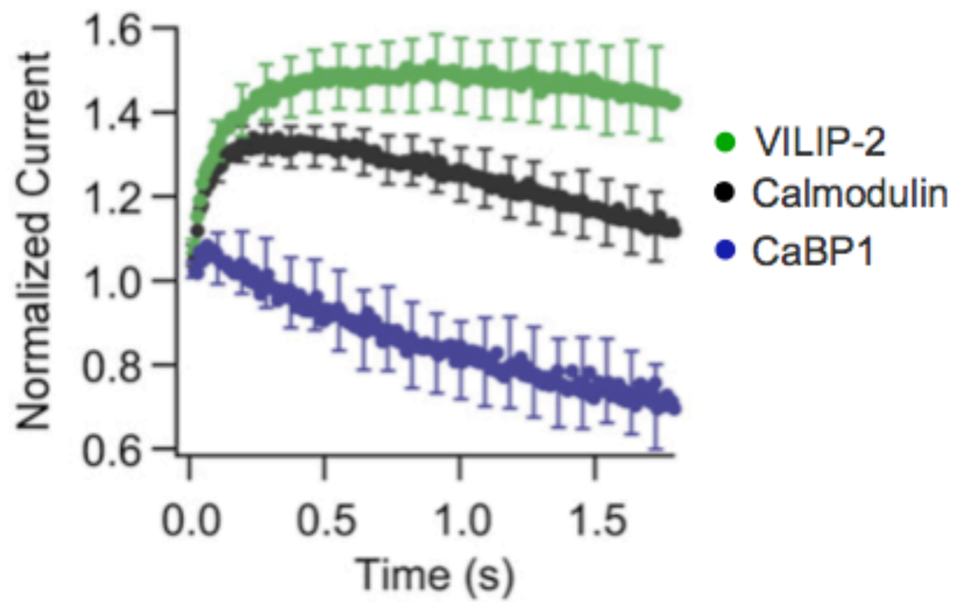


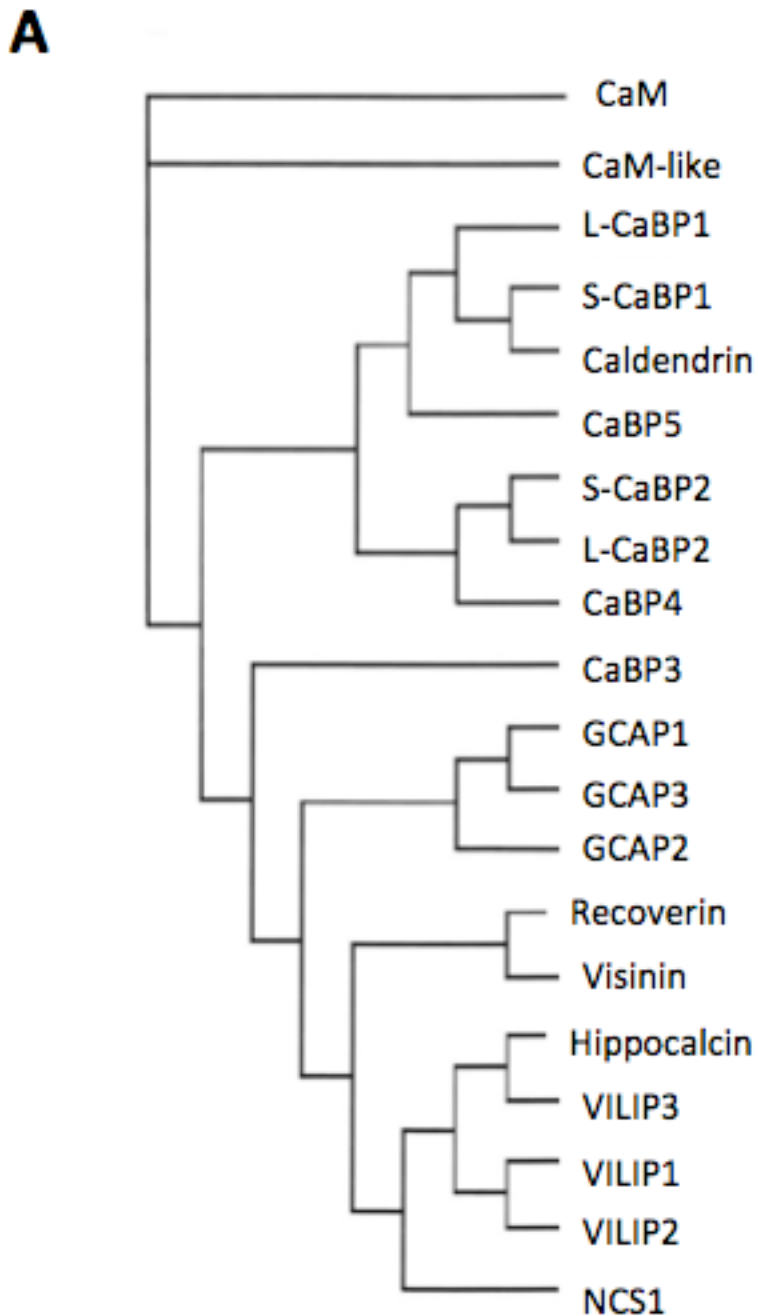


Figure 1.3 Phylogenetic tree of neuronal CaS protein super family

(A) The members of the neuronal CaS protein family expressed in mammalian species. CaBPs show 50-65% identity with CaM, in contrast VILIPs show 25-35% sequence identity with CaM (Haeseleer et al., 2000; Paterlini et al., 2000).

Chapter II

Differential regulation of $Ca_V2.1$ channels by Ca^{2+} sensor protein CaBP1 in synaptic transmission

Summary

Modulation of P/Q-type Ca^{2+} currents through presynaptic $Ca_V2.1$ channels by binding of Ca^{2+}/CaM contributes to short-term synaptic plasticity. CaBP1, a neurospecific CaM-like CaS protein differentially modulates $Ca_V2.1$ channels, but how it contributes to short-term synaptic plasticity is unknown. Here, we show that activity-dependent modulation of presynaptic $Ca_V2.1$ channels by CaBP1 has opposing effects on short-term synaptic plasticity in SCG neurons compared to endogenous CaM. In non-neuronal cells, CaBP1 prevents Ca^{2+}-dependent facilitation of P/Q-type Ca^{2+} current in transfected $Ca_V2.1$ channels. Overexpression of CaBP1 in SCG neurons displaces endogenous CaM from shared binding sites and alters synaptic transmission. CaBP1 markedly reduced synaptic facilitation during paired-pulses and trains of activity compared to endogenous CaM regulation of $Ca_V2.1$ channels. This effect of CaBP1 is dependent on binding to CaS regulatory sites at the C-terminus of the channel. These results demonstrate that activity-dependent regulation of presynaptic $Ca_V2.1$ channels by differentially expressed CaS proteins can fine-tune synaptic responses to trains of action potentials and thereby contribute to the diversity of short-term synaptic plasticity.

Introduction

Short-term facilitation of synaptic release has historically been attributed to enhanced vesicle release resulting from the accumulation of intracellular Ca^{2+} in the presynaptic terminal during repetitive action potentials (APs), in which the buildup of residual Ca^{2+} enhances binding to an unknown Ca^{2+} sensor that directly regulates neurotransmitter release (Katz and Miledi, 1968). Short-term depression of synaptic release has been traditionally attributed to vesicle depletion (Zucker and Regehr, 2002). However, at the large calyx of Held synapse, it has been shown that short-term synaptic plasticity is correlated with regulation of presynaptic $Ca_V2.1$ channels (Cuttle et al., 1998; Tsujimoto et al., 2002; Xu and Wu, 2005). Further, a recent study using recombinant $Ca_V2.1$ channels expressed in cultured superior cervical ganglion (SCG) neurons demonstrated that the Ca^{2+}-dependent facilitation (CDF) and Ca^{2+}-dependent inactivation (CDI) of Cav2.1 channels are mediated through neuronal Ca^{2+} sensor proteins (CaS) that bind the C-terminus and induce short-term facilitation and rapid synaptic depression, respectively (Mochida et al., 20008).

In non-neuronal cells, $Ca_V2.1$ channels undergo dual feedback regulation by Ca^{2+} that is mediated by calmodulin (CaM). CDF and CDI are mediated by binding of Ca^{2+}/CaM to a bipartite site in the C-terminal domain of the $Ca_V2.1$ channel $\alpha1$ subunit composed of an IQ-like motif (IM) and a CaM-binding domain (CBD) (DeMaria et al., 2001; Lee et al., 1999; Lee et al., 2003; Liang et al., 2003). Mutation of the IM site (IM-AA) abolishes CDF, whereas deletion of the CBD (△CBD) reduces inactivation and preserves facilitation (DeMaria et al., 2001; Lee et al., 2003; Mochida et al., 2008). In transfected SCG neuron synapses, mutations of the IQ-like domain and deletion of the CBD block synaptic facilitation and depression, respectively (Mochida et al., 2008).

Short-term synaptic plasticity is diverse at different synapses in the central nervous system. Because CaM is ubiquitously expressed, other proteins must determine the diversity of short-term synaptic plasticity. Ca^{2+}-binding proteins (CaBPs) are members of the superfamily of the neuronal CaS protein family homologous to CaM that have the ability to replace CaM at certain synapses, such as cerebellar and hippocampal neurons (Lee et al., 2002; Zhou et al., 2004), photoreceptor synapses (Haeseleer et al., 2000), and auditory hair cells (Cui et al., 2007; Yang et al., 2006). CaS proteins are unique in that Ca^{2+} binding promotes conformational changes, unlike many proteins whose function is simply to buffer excess free Ca^{2+} that do not undergo changes in conformation upon Ca^{2+} binding (Blatow et al., 2003). The family of CaBPs regulates a wide variety of voltage- and ligand- gated ion channels (Braunewell and Gundelfinger, 1999; Calin-Jageman and Lee, 2008; Haeseleer et al., 2000). For voltage-gated Ca^{2+} channels, Ca^{2+}-binding proteins can differentially regulate specific channel properties of inactivation, activation, open probability (P_o), Ca^{2+}-dependent facilitation, and surface expression (Lee et al., 2002; Cui et al., 2007; Few et al., 2005; Lautermilch et al., 2005; Yang et al., 2006; Zhou et al., 2004). These diverse forms of regulation by CaBPs may expand not only the functional range of Ca_V channels but also synaptic transmission.

CaBP1 is a Ca^{2+}-binding protein enriched in the brain and retina and has been found to be a prominent regulator of presynaptic $Ca_V2.1$ channels affecting Ca^{2+}-dependent facilitation (Few et al., 2005; Few et al., 2012; Lee et al., 2002). As with many neuronal CaS proteins, both CaBP1 and CaM comprise two lobes that bear a pair of EF-hand Ca^{2+}-binding motifs (Haeseleer et al., 2002). Beyond this common architecture, there are a number of differences. CaBP1 is myristoylated at its N terminus, has an N-lobe that is partially

insensitive to calcium (K_d > 100 μM) at EF-hand position 2 (Wingard et al., 2005) and a longer interlobe linker (Haeseleer et al., 2002).

Functionally, CaBP1 differentially affects $Ca_V2.1$ channels in transfected non-neuronal cells. CaBP1 enhances inactivation, causes a depolarizing shift in the voltage dependence of activation, and does not support Ca^{2+}-dependent facilitation of $Ca_V2.1$ channels (Lee et al., 2002). These inhibitory effects of CaBP1 do not require Ca^{2+} binding, but depend on the CBD in the α1 subunit of $Ca_V2.1$ channels (Lee et al., 2002; Few et al., 2012). CaBP1 binds to the CBD, co-immunoprecipitates with $Ca_V2.1$ channels from transfected cells and brain extracts, and colocalizes with $Ca_V2.1$ channels in discrete microdomains of neurons in the hippocampus and cerebellum (Lee et al., 2002). These data suggest that an interaction between $Ca_V2.1$ channels and CaBP1 may regulate Ca^{2+}-dependent forms of synaptic plasticity by inhibiting Ca^{2+} influx into neurons. Because of the nonlinear relationship between Ca^{2+} entry and neurotransmitter release regulation $Ca_V2.1$ channels would provide an efficient way to modulate release (Dodge and Rahamimoff, 1967a).

Moreover, CaBP1 can substitute for CaM in vitro, which suggests that neuronal CaS proteins may regulate effectors that are typically thought to be modulated by CaM (Lee et al., 2002). Given the widespread distribution of $Ca_V2.1$ channels throughout the nervous system, the cell type–specific modulation of $Ca_V2.1$ by CaBP1 may fundamentally determine the nature of presynaptic Ca^{2+} signals and the functional output of synaptic activity. How CaBP1 regulation of $Ca_V2.1$ channels contributes to short-term synaptic plasticity is unknown. In this study, we use a model synapse to express CaBP1 presynaptically with $Ca_V2.1$ channels to probe the functional properties of CaBP1 regulation in synaptic transmission. $Ca_V2.1$

channels can be functionally expressed in cultured sympathetic superior cervical ganglion (SCG) neurons, which do not express endogenous $Ca_V2.1$ channels (Mochida et al., 2003). Transfected SCG neurons form fast cholinergic synapses whose neurotransmission is solely by the heterologously expressed $Ca_V2.1$ channels after pharmacological block of endogenous $Ca_V2.2$ channels (Mochida et al., 2008; Mochida et al., 2003). Coexpression of CaBP1 and $Ca_V2.1$ channels significantly reduces synaptic facilitation. CaBP1 effect is dependent on direct binding to CaS binding sites on the C-terminus of $Ca_V2.1$ channels. These results further define the functional role of CaS protein-dependent modulation of short-term synaptic plasticity.

Experimental Methods

Expression of $Ca_V2.1$ in Cultured SCG Neurons

SCG neurons were cultured as described to allow synapse formation (Mochida et al., 1996). cDNAs encoding WT $\alpha_1 2.1$ subunit, double mutant (dMut) $\alpha_1 2.1_{IMAA/\Delta CBD}$ containing mutations in the IQ-like domain and calmodulin-binding domain, CaBP1and eGFP were microinjected into the nuclei of SCG neurons through glass micropipettes with 5% fast-green dye (Sigma-Aldrich, St Louis, MO). Entry of the constructs into the cell nucleus was monitored by the intensity of green dye in the nucleus. The cells were maintained at 37 ^oC in a 95% air, 5% CO_2-humidified incubator for 48 hours, after which, the injected neurons were identified with an inverted microscope (Olympus) equipped with an epifluorescence unit.

Synaptic Transmission Between SCG Neurons

Excitatory postsynaptic potentials (EPSPs) were recorded from SCG neurons cultured for 6 weeks as described (Mochida et al., 1996). Conventional intracellular membrane potential

recordings were made from two neighboring neurons using microelectrodes filled with 1 M KAc (70-90 MΩ). EPSPs were recorded from a non-injected neuron while action potentials were generated in the injected, presynaptic neuron expressing the $\alpha_1$2.1 channels and CaS protein by passing 1-2 nA of current for 5 ms through an intracellular recording electrode (Intracellular Electrometer IE-210, Warner Instruments, Hamden, CT). All endogenous synaptic transmission was blocked by bath application of 3 μM ω-conotoxin GVIA in a modified Krebs' solution consisting of 136 mM NaCl, 5.9 mM KCl, 1 mM $CaCl_2$, 1.2 mM $MgCl_2$, 11 mM glucose, and 3 mM Na-HEPES (pH 7.4). For recording sub-threshold EPSPs, the membrane potential of the postsynaptic cell was held at -70 or -80 mV by passing current (0.2-0.4 nA) through the recording electrode. Electrophysiological data were collected using PULSE (Heka Electronik, Lambrecht, Germany) and analyzed with Igor Pro (Wavemetrics, Lake Oswego, OR). Each protocol was repeated 10 times and averaged for each synapse. The EPSP amplitudes were averaged to account for variation in transmitter release following repetitive action potentials. The peak amplitudes from baseline were averaged and normalized to the first EPSP of each train and plotted against action potential number. Data values with associated error shown in the text and figures represent mean ± S.E.M. Statistical significance for comparisons of the various expression vectors were performed using one-way ANOVA followed by Tukey's post test denoted by + and post hoc Bonferroni test denoted by *. Mean data for EPSP trains (Figures 2.3 and Figure 2.4) were fit with bi-exponential functions $C+A\exp(-t/\tau1)+(1-A-C)\exp(-t/\ \tau2)$. $\tau1$ describing facilitation and the absolute value were poorly defined by the data and fixed at reasonable values for fitting. Best-fit lines (solid) and 95% confidence estimates for these fits (dotted lines) are provided in the figures.

Patch Clamp Recordings in SCG Neurons

SCG neurons were injected one week after dissociation and plating. cDNAs encoding WT $\alpha_1$2.1 subunit and eGFP, with or without CaBP1, were microinjected into the nuclei of SCG neurons through glass micropipettes with 5% fast-green dye (Sigma-Aldrich, St Louis, MO). Entry of the constructs into the cell nucleus was monitored by the intensity of green dye in the nucleus. The cells were maintained at 37 ^{o}C in a 95% air, 5% CO_2-humidified incubator for 24 hours, after which, the injected neurons were identified with an inverted microscope (Olympus) equipped with an epifluorescence unit. For whole-cell voltage clamp recordings, patch pipettes of borosilicate glass with resistances of 3-6 MΩ were used to record P/Q-type Ca^{2+} currents from injected SCG neurons. Pipettes were filled with intracellular solution containing (in mM) N-methyl-D-glucamine (125), TEA-Cl (10), HEPES (10), $MgCl_2$ (1), EGTA (0.5), MgATP (4), and Na_2GTP (0.3), and Tris-creatine phosphate (14) adjusted to pH 7.2 with methanesulfonic acid. The extracellular solution contained (in mM) TEA-Cl (141.9), $MgCl_2$ (1.2), HEPES (3), and $CaCl_2$ (1) adjusted to pH 7.4 with NaOH. P/Q-type Ca^{2+} currents were isolated by bath application of (in µM) nifedipine (10), SNX-482 (0.1), ω-conotoxin GVIA (3), and TTX (1) to block L-, R-, and N-type Ca^{2+} currents and sodium currents, respectively. Recordings were obtained using an HEKA EPC10 amplifier (HEKA Elektronik, Lambrecht, Germany) with PULSE software and filtered at 5 kHz. All currents were generated by depolarizing from a holding potential of -80 mV. Leak and capacitive transients were subtracted using a P/-4 protocol. Data was analyzed using Igor Pro (Wavemetrics, Lake Oswego, OR). All averaged data and error bars represent the mean ± S.E.M.

Results

CaBP1 Reduces Paired-pulse Facilitation

CaBP1 enhances inactivation and does not support Ca^{2+}-dependent facilitation of $Ca_V2.1$ channels (Lautermilch et al., 2005; Lee et al., 2002). In order to determine whether presynaptic expression of these CaS proteins affects short-term synaptic plasticity in SCG neurons, we designed our experiments to measure synaptic transmission driven by $Ca_V2.1$ channels expressed only on the presynaptic side of the synapse, and we blocked the endogenous N-type Ca^{2+} current with ω-conotoxin GVIA. Under these conditions, synaptic transmission is mediated by transfected $Ca_V2.1$ channels specifically (Mochida et al., 2008; Mochida et al., 2003). We expressed $Ca_V2.1$ channels and CaBP1 by microinjection of cDNA into an identified SCG neuron, and we recorded excitatory postsynaptic potentials (EPSPs) from a neighboring synaptically connected, but untransfected neuron, thereby isolating presynaptic effects.

Paired pulse stimuli are a simple approach to probe synaptic plasticity. When two action potentials are generated in rapid succession, the postsynaptic response to the second is often larger due to paired-pulse facilitation (PPF). This facilitation is thought to result from increased residual Ca^{2+} remaining in the presynaptic terminal from the first action potential, but other mechanisms may also contribute (Inchauspe et al., 2004; Mochida et al., 2008; Xu and Wu, 2005; Zucker, 1993). PPF is defined by a paired pulse ratio (PPR) larger than 1, whilst paired pulse depression (PPD) is defined by a PPR < 1. We first tested synaptic transmission under conditions of low release probability (1 mM extracellular Ca^{2+}) where facilitation is observed in control synapses using a paired-pulse protocol. In response to paired stimuli, synapses expressing WT $Ca_V2.1$ channels showed much larger paired-pulse

ratio (PPR) compared to CaBP1 synapses, across a range of interstimulus intervals (ISIs) (Figures 2.1*B* and 2.1*C*). For synapses expressing WT $Ca_V2.1$ channels we observed paired-pulse facilitation (PPF) at ISIs beginning at 50 ms consistent with previous findings (Figure 2.1*A* and 2.1*B*) (Mochida et al., 2008). These results are similar to PPF and PPD recorded at synapses between central neurons at which transmission is initiated by P/Q-type Ca^{2+} currents (Ishikawa et al., 2005). In contrast, CaBP1-expressing synapses showed PPD rather than PPF at ISI tested up to 150 ms. When the second stimulus followed the first by a few milliseconds (Figure 2.1*A*), the second EPSP response was smaller than the first. When the interval between stimuli was over than 150 ms, however, the second response recovered and was comparable in size to the first EPSP (Figure 2.1*B*). At 80 ms ISI, where we obtained maximal PPF for synapses expressing WT $Ca_V2.1$ channels compared to CaBP1 co-expressing synapses showed strong depression (Figure 2.1*C* ; WT PPR_{80ms} 1.36 $\pm$ 0.12 (n=23); CaBP1 PPR_{80ms}, 0.76 $\pm$ 0.12 (n=17), $^{**}p < 0.05$, $^{*}p < 0.01$, Bonferonni corrected t-test; ++ $p < 0.05$, $+p < 0.01$, post hoc Tukey's t-test). At all ISI tested up to 200 ms, expression of CaBP1 led to a reliable decrease in the magnitude of the PPR at all synapses.

Immunocytochemistry studies show that CaBP1 co-localizes with $Ca_V2.1$ channels and syntaxin in the CA1 region of the hippocampus and in the molecular layer of the cerebellum, although postsynaptic staining has also been observed (Lee et al., 2002). Since CaBP1 is only expressed in the transfected presynaptic neuron in our SCG synapses, its effect is limited to presynaptic mechanisms involved in synaptic plasticity. However, it is possible that CaBP1 may act on presynaptic machinery involved in neurotransmitter release other than $Ca_V2.1$ channels. To determine if the decrease in PPR is due to changes in basal release probability we compared the initial EPSP for WT $Ca_V2.1$ channel expressing synapses and synapses

expressing CaBP1. Expression of CaBP1 did not change the amplitude of the first synaptic response in a paired stimulus compared to WT (WT=2.97 $\pm$ 0.22 mV (n=20); WT+CaBP1= 3.27 $\pm$ 0.41 mV (n=16), $p > 0.01$). Based on these results, we conclude that CaBP1 expression does not affect initial release probability. Additionally, to confirm the decrease in PPR is not due to changes in expression of various CaS proteins or a reduction in Ca^{2+} influx through presynaptic Ca^{2+} channels, we compared whole-cell Ca^{2+} currents recorded from WT $Ca_V2.1$ channels and CaBP1 expressing SCG somas. We observed no significant difference in Ca^{2+} current amplitudes recorded by whole-cell voltage clamp in transfected SCG neuronal cell bodies (Figure 2.1*D*). Our data imply that CaBP1 binds Ca^{2+} entering through $Ca_V2.1$ channels and blocks facilitation of the Ca^{2+} current, enhancing inactivation and resulting in reduced PPF (Lee et al., 2002). These data indicate that CaS-dependent inactivation of $Ca_V2.1$ channels by CaBP1 causes PPD that opposes synaptic facilitation. Thus, a switch from facilitation to depression was evident in CaBP1-expressing synapses by changing release probability in an activity-dependent manner in response to Ca^{2+} entry during the first action potential of the pair.

CaBP1 Binding to $Ca_V2.1$ Channels is Essential for Modulation of Paired-Pulse Facilitation

Recent studies at some synapses suggest that short-term depression, particularly PPD, is independent of depletion (Bellingham and Walmsley, 1999; Brody and Yue, 2000; Kraushaar and Jonas, 2000; Waldeck et al., 2000; Xu and Wu, 2005; Sullivan, 2007). Does CaBP1 bind directly to $Ca_V2.1$ channels to block synaptic facilitation resulting in synaptic depression? The interaction between CaBP1 and $Ca_V2.1$ channels requires the same

intracellular domain of $Ca_V2.1$ that binds CaM. CaBP1 predominantly binds to the CBD of $Ca_V2.1$ channels and mutation of the IM site and deletion of the CBD abolishes CaBP1 induced fast inactivation of Ca^{2+} currents in transfected non-neuronal cells (Lee et al., 2002). To address whether CaBP1 acts directly on $Ca_V2.1$ channels, we transfected SCG neurons with mutant $Ca_V2.1$ channels lacking CaS binding domains, IM-AA/△CBD. Co-expression of CaBP1 had no effect on these mutant $Ca_V2.1_{IM-AA/\triangle CBD}$ channels (dMut) in paired-pulse experiments (Figure 2.2*A*-2.2*C*). $Ca_V2.1_{IM-AA/\triangle CBD}$ expressing synapses showed much less PPF at intermediate ISI compared to WT $Ca_V2.1$ channels (WT, Figure 2.1*B*; dMut, Figure 2.2*B*). The very low level of PPF remaining in synapses expressing the dMut channel likely reflects a CaS-independent facilitation mechanism. Moreover, co-expression of CaBP1 did not affect PPR observed for any ISI tested compared to dMut $Ca_V2.1_{IM-AA/\triangle CBD}$ channels (dMut PPR_{50ms}=1.13 $\pm$ 0.183, +CaBP1 PPR_{50ms} = 1.05 $\pm$ 0.105; dMut PPR_{80ms} 0.836 $\pm$ 0.121, +CaBP1 PPR_{80ms} = 0.950 $\pm$ 0.157; dMut PPR_{150ms} =0.923 $\pm$ 0.125, +CaBP1 PPR_{150ms}=0.981 $\pm$0.189; Figure 2.2*B* and 2.2*C*, $p > 0.1$). These results confirm that the ability of CaBP1 to reduce PPF results from its binding to the CaS binding site on $Ca_V2.1$ channels. Since basal release probability for WT and CaBP1 expressing synapses remained unchanged and expression of $Ca_V2.1$ channels lacking CaS-binding sites abolished the CaBP1 affect, this provides strong evidence that the effect of CaBP1 is to regulate $Ca_V2.1$ channels and presynaptic Ca^{2+} currents.

CaBP1 Reduces Synaptic Facilitation During Trains of Activity

During repetitive activation, certain excitatory synapses in the brain exhibit prominent frequency-dependent synaptic facilitation followed by depression. Activity-

dependent increases in Ca^{2+} entry cause facilitation followed by inactivation of $Ca_V2.1$ channel currents that correlates with synaptic output (Borst and Sakmann, 1998b; Cuttle et al., 1998; Inchauspe et al., 2004; Ishikawa et al., 2005; Lee et al., 2000; Lee et al., 1999; Xu and Wu, 2005). This dual regulation of Ca^{2+} currents is caused by sequential binding of Ca^{2+}/CaM binding to the IQ-like domain and CBD of $Ca_V2.1$ channels (DeMaria et al., 2001; Lee et al., 1999; Lee et al., 2003). To investigate the effects of CaBP1 on $Ca_V2.1$ channels during trains of activity, we stimulated synapses at varying frequencies and recorded EPSPs during each stimulation in the presence of 1 mM extracellular Ca^{2+} to give a low basal probability of neurotransmitter release (Figure 2.3*A* and Figure 2.3*B*).

In control synapses expressing WT $Ca_V2.1$ channels alone, we observed synaptic facilitation that then decayed at all stimulus frequencies (Figure 2.3*B*). In order to include all of the data points in our statistical analysis, the mean synaptic responses were fit to a bi-exponential function, the time constant for depression was derived from this fit, and the value for maximum facilitation was estimated by extrapolation to time of the second stimulus in the train. Errors in the fits and parameter estimates were expressed as 95% confidence limits (Figure 2.3*B*, dotted lines). This profile of synaptic facilitation and depression resembles regulation of $Ca_V2.1$ channels by endogenous CaM (Figure 2.3*B*) (Mochida et al., 2008). Synaptic facilitation for WT synapses increased as stimulus frequency increased. In contrast, synapses expressing CaBP1 showed less facilitation and increased depression at each stimulus frequency tested beginning at 10 Hz (Figure 2.3*B*). Peak synaptic facilitation was reduced on average from 2.22 ± 0.25 to 1.53 ± 0.15 at 30 Hz ($p < 0.05$) and EPSP amplitude after the peak response also showed enhanced depression compared to WT control synapses (Figure 2.3*B*). CaBP1 significantly reduces synaptic facilitation at $Ca_V2.1$ mediated synapses.

These data complement published research showing that CaBP1 does not support Ca^{2+}-dependent facilitation of $Ca_V2.1$ channels during trains of activity. We conclude that CaBP1-expressing synapses have reduced synaptic facilitation during trains of activity under conditions of low release probability and enhanced inactivation.

CaBP1 Binding to $Ca_V2.1$ Channels is Required for Its Effects on Synaptic Plasticity in Trains of Stimuli

In order to test whether direct binding of CaBP1 to $Ca_V2.1$ channels is required for modulation of synaptic facilitation during trains of stimuli, we expressed CaBP1 with the double mutant $Ca_V2.1_{IM\text{-}AA/\triangle CBD}$ (dMut). SCG neurons expressing dMut $Ca_V2.1_{IM\text{-}AA/\triangle CBD}$ show peak synaptic facilitation that is reduced from 2.22±0.21 to 1.48±0.12 at 30 Hz compared to synapses expressing WT $Ca_V2.1$ channels (Figure 2.4*B* compared to Figure 2.3*B*, $p<0.05$). dMut $Ca_V2.1_{IM\text{-}AA/\triangle CBD}$ synapses at 10 Hz showed no synaptic facilitation at initial pulses, however synaptic facilitation recovered at frequencies 20 Hz and 30 Hz during the first five pulses of the train (Figure 2.4*B*). These results are consistent with the previous conclusion that normal synaptic facilitation in this model synapse requires facilitation of $Ca_V2.1$ channels via CaS proteins interacting with the IM and CBD domains (Mochida et al., 2008). Co-expression of CaBP1 does not further reduce facilitation of the mutant $Ca_V2.1_{IM\text{-}AA/\triangle CBD}$ during trains of action potentials (Figure 3.4*B*). Taken together, these data indicate that mutation of CaS binding sites eliminates regulation by CaBP1 as seen in paired-pulses and activity-dependent plasticity, and therefore show that direct binding of CaBP1 to $Ca_V2.1$ channels is sufficient to cause a switch from synaptic facilitation to depression.

Discussion

Our results provide new insights into the role of CaBP1 in the regulation of neuronal Ca^{2+} signaling and synaptic transmission. CaBP1 binds to the same sites as CaM in the C-terminal domain of the α1subunit of $Ca_V2.1$ channels but has opposite effects on channel function and consequently on synaptic plasticity. We show that it is possible to alter short-term plasticity by expressing CaBP1 and $Ca_V2.1$ channels at the synapse in SCG neurons. CaBP1 significantly reduced synaptic facilitation during paired-pulses and trains of activity compared to endogenous CaM regulation of $Ca_V2.1$ channels. This effect of CaBP1 is dependent on binding to CaS regulatory sites at the C-terminus of the channel. Mutation of the IM site (IM-AA) and additional deletion of the CBD abolished CaBP1 effects. These results indicate that the reduction of presynaptic Ca^{2+} entry by inactivation of presynaptic $Ca_V2.1$ channels caused by CaS proteins bound to the IM site and CBD is the major cause of synaptic depression during short bursts of activity at frequencies up to 30 Hz at this synapse. Thus, activity-dependent regulation of presynaptic $Ca_V2.1$ channels by differentially expressed CaS proteins can fine-tune synaptic responses to trains of action potentials and thereby contribute to the diversity of short-term synaptic plasticity.

Synaptic facilitation and depression have been studied for decades, but the molecular mechanisms have remained elusive (Zucker and Regehr, 2002). Accordingly, it has been suggested that regulation of presynaptic Ca^{2+} channels is an economic way to achieve various forms of synaptic plasticity since neurotransmitter release is highly nonlinearly related to Ca^{2+} influx (Neher, 1998). Recent studies at the giant calyx of Held synapse reveal that regulation of Ca^{2+} channels, particularly $Ca_V2.1$ channels, in nerve terminals contributes significantly to synaptic facilitation and depression (Xu and Wu, 2005; Xu et al., 2007; Borst

and Sakmann, 1998). Both short-term facilitation and the rapid component of depression of synaptic transmission are correlated with the amplitude and kinetics of Ca^{2+}-dependent facilitation and inactivation of $Ca_V2.1$ channel current (Ishikawa et al., 2005; Mochida et al., 2008; Forsythe et al., 1998; Xu and Wu, 2005).

We show evidence that CaBP1 can act in a push-pull manner to set the sign of short-term plasticity, facilitation versus depression. Our previous results with cultured SCG neuron synapses driven by transfected $Ca_V2.1$ channels reveal that CaS-dependent regulation of presynaptic Ca^{2+} entry, mediated by CaS interactions with the IQ-like domain and the CBD in the C-terminus of $Ca_V2.1$ channels, makes a major contribution to short-term plasticity, both synaptic facilitation and depression (Mochida et al., 2008). Here we show that CaBP1, a neuronal CaS protein capable of substituting for CaM, interacts with these regulatory sites and modulates $Ca_V2.1$ channels by limiting Ca^{2+} influx during activity dependent plasticity. Thus, CaBP1 may potentially diversify synaptic transmission and plasticity in a cell-specific manner.

Interaction of CaBP1 with the CBD $Ca_V2.1$ channels expressed in non-neuronal cells positively shifts the voltage dependence of activation of $Ca_V2.1$ channels, supports faster inactivation in both single depolarizations and trains of depolarizations, and reduces Ca^{2+}-dependent facilitation (Lee et al., 2002). At the calyx of Held, Ca^{2+}/CaM-mediated Ca^{2+} current inactivation is a primary source of short-term depression (Xu and Wu, 2005). Application of potent CaM inhibitors partially relieved presynaptic Ca^{2+} current inactivation in response to repetitive action potential trains reducing synaptic depression (Xu and Wu, 2005). However, the relief of inactivation of the Ca^{2+} current by CaM inhibitors was partial suggesting the involvement of other CaS-dependent mechanisms. Our results provide the first

direct evidence to our knowledge that CaS-dependent inactivation of presynaptic $Ca_V2.1$ channels by direct binding of a specific CaS protein different from CaM, CaBP1, can mediate short-term plasticity. Expression of CaBP1 in SCG synapses markedly reduces synaptic facilitation and enhances rapid synaptic depression when studied under conditions of low release probability in the presence of 1 mM extracellular Ca^{2+}, where synaptic facilitation is prominent while not affecting basal release probability (Figure 2.1*B* and Figure 2.3*B*). These effects of CaBP1 were abolished when $Ca_V2.1$ channels lacking essential CaS binding sites were coexpressed at SCG synapses. At these synapses, the postsynaptic response is therefore controlled by CaS-dependent modulation of presynaptic Ca^{2+} entry. The reduction of presynaptic Ca^{2+} entry by inactivation of presynaptic Ca^{2+} currents caused by CaS proteins bound to the CBD is a major cause of synaptic depression during short bursts (up to 1 s) of activity at frequencies up to 30 Hz at this synapse. Inactivation is a negative feedback process that prevents excessive Ca^{2+} entry via Ca^{2+} channels and may be regulated by CaBP1 in neurons. The mechanism that causes short-term depression without depletion is debated. It has been attributed to a decreased release probability of vesicles in the readily releasable pool (RRP) during an action potential (Betz, 1970). Studies have shown that except during a 100 Hz AP train, inactivation of Ca^{2+} currents, but not depletion, was the dominant mechanism contributing to short-term synaptic depression (Xu and Wu, 2005). Our results further support the notion that Ca^{2+}/CaS-dependent inactivation of $Ca_V2.1$ channels contributes to synaptic depression. CaBP1 may displace CaM from shared binding sites and affect Ca^{2+} consequently altering neurotransmitter release in an activity-dependent manner.

Increasing evidence supports a role for neuronal CaS proteins, such as CaBP1, in diversifying the properties of voltage-gated Ca^{2+} channels in neurons (Wang et al., 2001;

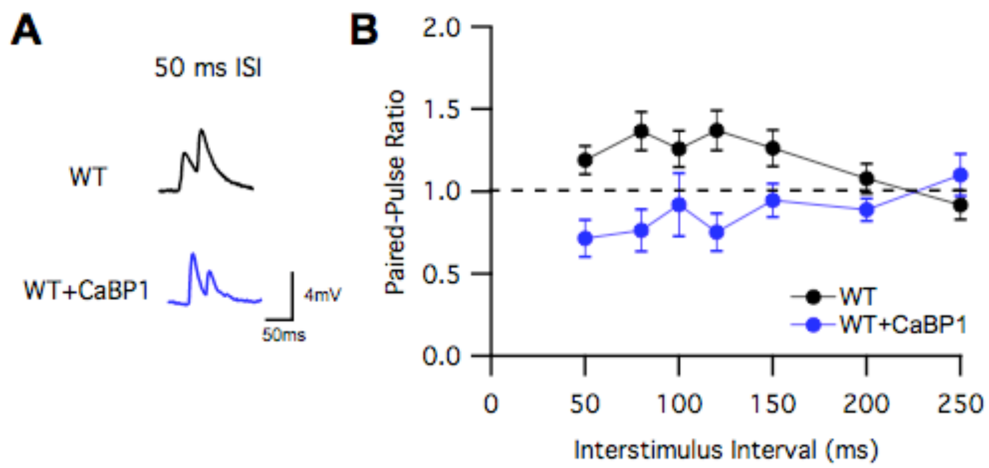
Weiss and Burgoyne, 2001; Lee et al., 2002; Tsujimoto et al., 2002; Weiss and Burgoyne, 2002). Given that CaM binding and regulation appears to be a common mechanism among different Ca^{2+} channel types (Liang et al., 2003), neuronal CaS proteins, like CaBP1, may contribute to the heterogeneous properties of these channels in neurons. In some cases, the modulatory effect of a neuronal CaS protein may be different to that of CaM or CaBP1. For example, VILIP-2 differentially regulates $Ca_V2.1$ channels by slowing the rate of inactivation and enhancing facilitation. Likewise, mutation of the IQ-like domain combined with deletion of the CBD prevent VILIP-2 binding to $Ca_V2.1$ channels (Lautermilch et al., 2005). VILIP-2 has been shown to affect synaptic transmission in SCG neurons via Ca^{2+}/CaS-dependent modulation of $Ca_V2.1$ channels (See Chapter II). CaBP1 has been shown to bind and modulate postsynaptic $Ca_V1.2$ channels, which mediate L-type current, in a distinct manner compared to $Ca_V2.1$ channels. Whereas CaBP1 enhances inactivation of $Ca_V2.1$ channels, $Ca_V1.2$ channels undergo Ca^{2+}-dependent facilitation mediated by CaBP1 binding to the C-terminus of the channel (Zhou et al., 2004). In this case, CaBP1 interactions with $Ca_V1.2$ channels may play an important role in amplifying postsynaptic Ca^{2+} signals.

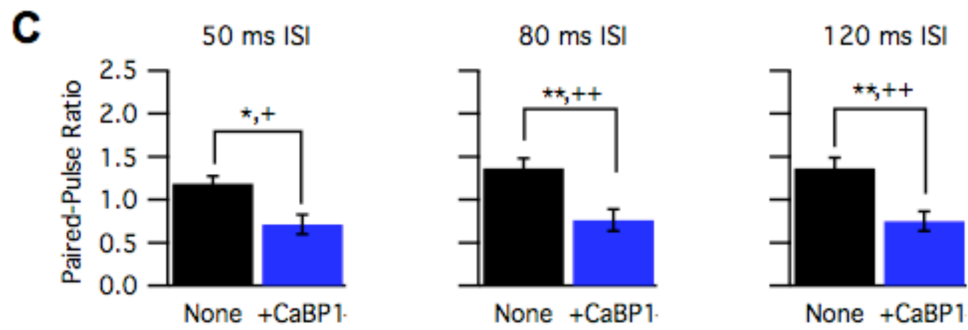
Although CaBP1 and CaM are ~50% similar at the amino acid level, CaBP1 differs in that it is N-terminally myristoylated, a modification that mediates its association with the plasma membrane (Haynes et al., 2004). Recent studies have identified the molecular determinants for differential regulation of $Ca_V2.1$ channels by CaBP1 through chimeric analysis in which the unique structural domains of CaBP1 were inserted into CaM. The N-terminal domain, including its myristoylation site, and the second EF-hand, which is inactive in Ca^{2+} binding, are the key molecular components of distinct regulation of $Ca_V2.1$ channels by CaBP1 (Few et al., 2005; Few et al., 2012). Whereas CaM binds Ca^{2+} ions with an affinity

of 5-10 μM, the Ca^{2+}-binding affinity for CaBP1 is considerably higher at 2.5 μM (Mikhaylova et al., 2011). Moreover, CaM is ubiquitously expressed throughout the brain, so cell-type specific expression of neuronal CaS proteins may provide a mechanism for synaptic diversity. CaBP1 is primarily expressed in cerebral cortex, retina, and hippocampus (Haeseleer et al., 2002). CaBP1 and $Ca_V2.1$ channels coexist at specialized synapses in the CA1 region of the hippocampus and in presynaptic terminals in the molecular layer of the cerebellum (Lee et al., 2002). Therefore, structural differences in EF-hand organization, variations in Ca^{2+}-binding affinity, distinct expression in neuronal subpopulations and, hence, distinct regulation of $Ca_V2.1$ channels make CaBP1 and neuronal CaS proteins plausible candidates to fine-tune Ca^{2+} signaling and synaptic transmission in neurons.

Figure 2.1. CaBP1 Reduces Paired-pulse Facilitation

(A) Representative EPSPs evoked by paired action potentials with 50-ms inter-stimulus interval in the presynaptic neurons expressing $Ca_V2.1$ alone (WT, black trace) and co-transfected with CaS protein CaBP1 (WT+CaBP1, blue trace) in the presence of ω-conotoxin GVIA (3 μM). **(B)** Average paired-pulse ratio (PPR) plotted against inter-stimulus interval (ISI) in milliseconds (ms) for WT (●), and WT+CaBP1 (●) in 1 mM extracellular Ca^{2+}. PPR equals the amplitude of the second EPSP divided by the amplitude of the first EPSP. **(C)** Paired-pulse ratios for 50 ms, 80 ms, and 120 ms inter-stimulus intervals for WT (black) and WT+CaBP1 (blue). * $p < 0.05$, ** $p < 0.01$, ANOVA with Bonferroni post-test. + $p < 0.05$, ++ $p < 0.01$, ANOVA with Tukey post-test for differences compared to WT group (absolute *p* values : ISI_{50ms} p=0.006, ISI_{80ms} p=0.008, and ISI_{120ms} p=0.0002). Averaged amplitudes (mean ± S.E.M.) of the first EPSP recorded for 50-ms ISI: WT, 2.97±0.22 mV (n=20) and WT+CaBP1, 3.27±0.41 mV (n=16; $p > 0.1$ vs. WT); **(A-C)** Data shown are mean ± S.E.M. from 10-20 synaptic pairs. **(D)** $Ca_V2.1$ channel Ca^{2+} currents in SCG neuronal cell bodies. CaS protein expression does not alter total Ca^{2+} channel current in transfected SCG soma. Current-voltage curves generated by eliciting P/Q-type Ca^{2+} currents with a series of depolarizing voltage-steps increasing by 5 mV increments for WT (●, n=8) and WT+CaBP1 (●, n=6) in 1mM Ca^{2+} external solution. WT+CaBP1 neurons show a shift in current activation as seen previously (Lee et al., 2002).


A
50 ms ISI
WT
WT+CaBP1
4mV
50ms
B
Paired-Pulse Ratio
Interstimulus Interval (ms)
WT
WT+CaBP1


C
50 ms ISI
80 ms ISI
120 ms ISI
Paired-Pulse Ratio
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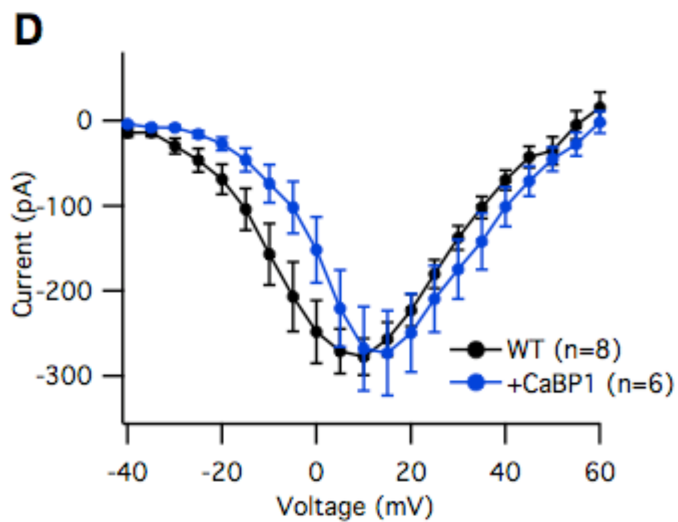
**,++
None
+CaBP1


D
Current (pA)
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WT (n=8)
+CaBP1 (n=6)

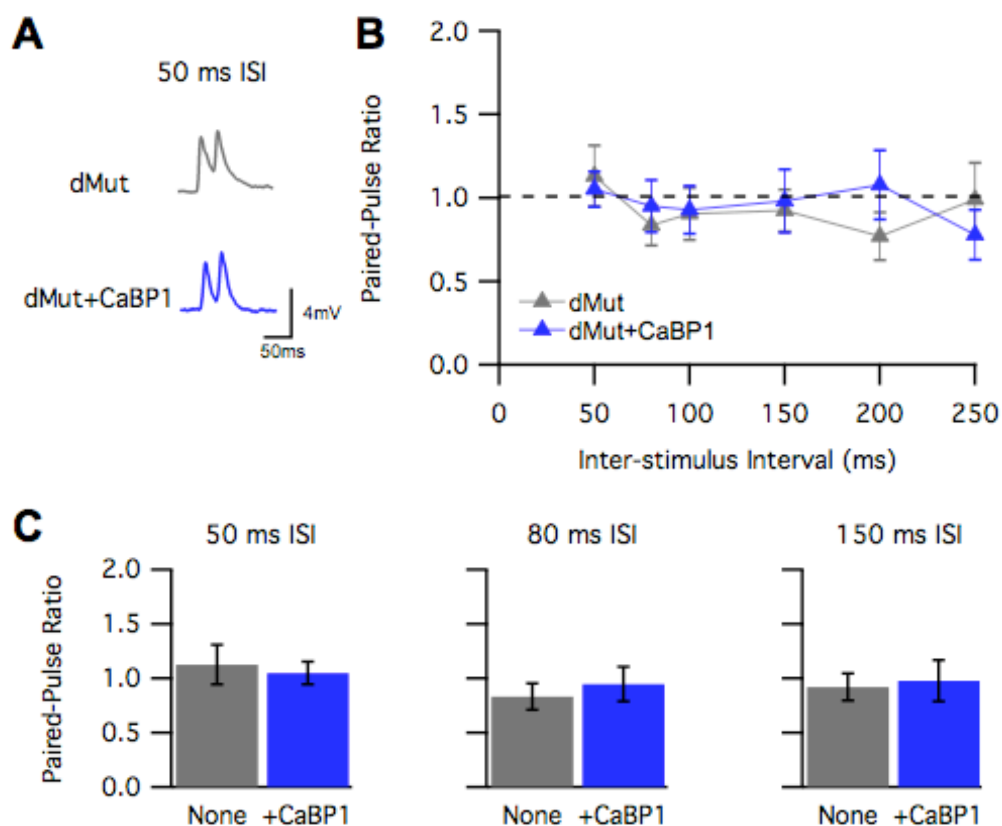


Figure 2.2. Mutation of the CaS Binding Site prevents CaBP1-mediated PPD

(A) Averaged EPSP traces (10 traces) elicited by neurons expressing $Ca_V2.1_{IMAA\text{-}\Delta CBD}$ alone (dMut, gray trace), or with CaS proteins CaBP1 (dMut+CaBP1, blue trace) evoked by paired-action potentials with 50 ms inter-stimulus interval in the presynaptic neurons. **(B)** Paired-pulse ratio (PPR) plotted against inter-stimulus interval (ISI) for dMut (▲) and dMut+CaBP1 (▲). PPR equals the amplitude of the second EPSP divided by the amplitude of the first EPSP. **(C)** Paired-pulse ratios for 50, 80, and 150 ms inter-stimulus intervals for dMut (gray) and dMut+CaBP1 (blue). Averaged first EPSP amplitude recorded for 50 ms ISI, dMut=6.6$\pm$1.6 (n=16), dMut+CaBP1=3.01$\pm$0.33 (n=14). **(A-C)** Data shown are mean $\pm$ S.E.M. from 10-15 synaptic pairs.

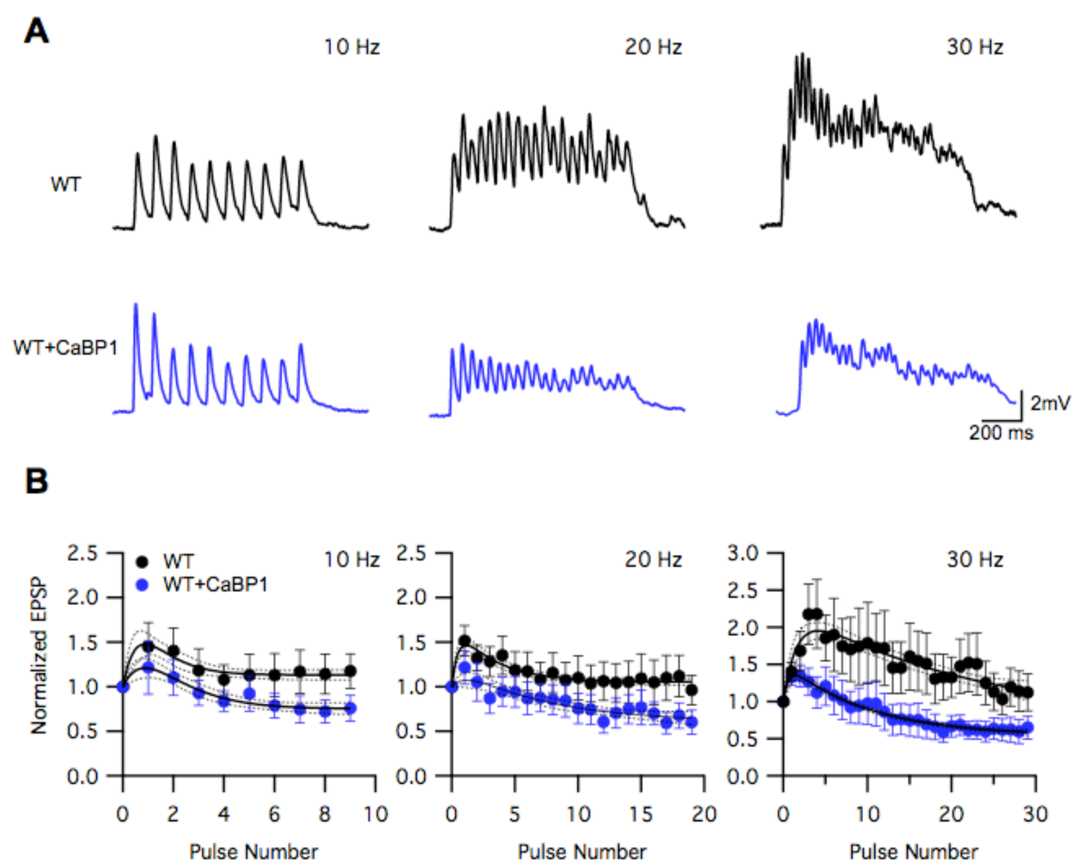


Figure 2.3. CaBP1 Reduces Synaptic Facilitation in Trains of Stimuli

(A) Representative EPSPs in 1 mM extracellular Ca^{2+} evoked by repetitive action potentials at 10, 20, and 30 Hz for 1s in the presynaptic neurons expressing wild-type $Ca_V2.1$ alone (WT, black) or co-transfected with CaBP1 (WT+CaBP1, blue). Data from 10 sweeps repeated every 30s at each frequency were averaged. **(B)** Mean normalized EPSP amplitude from 10-16 synaptic pairs at 10, 20, and 30 Hz frequency (5 Hz not shown). EPSP amplitudes were normalized to the first EPSP of each train and plotted against action potential number for WT (●) or WT+CaBP1 (●). Points represent the mean $\pm$ S.E.M. Solid lines are best-fits of a bi-exponential equation as described in Experimental Procedures with the 95% confidence intervals of the fits indicated by dotted lines.

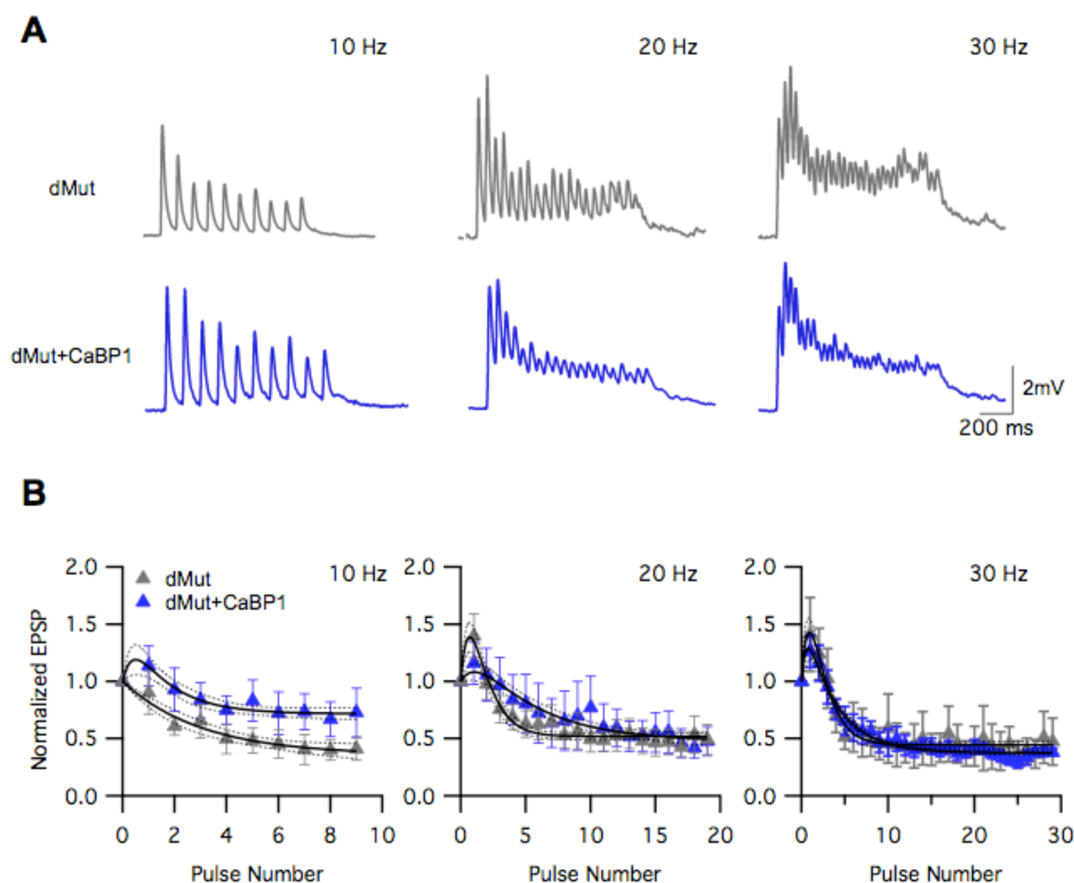


Figure 2.4. Mutation of the CaS Binding Site Reduces Modulation of Short-term Synaptic Plasticity by CaBP1

(A) Representative EPSPs in 1 mM extracellular Ca^{2+} evoked by repetitive action potentials at 10, 20, and 30 Hz for 1s in the presynaptic neurons expressing $Ca_V2.1_{IMAA\text{-}\Delta CBD}$ alone (dMut, gray trace), or with CaS proteins CaBP1 (dMut+CaBP1, blue trace). Data from 10 sweeps repeated every 30s at each frequency were averaged. **(B)** Normalized EPSP amplitudes for $Ca_V2.1$ channel with mutations IMAA-ΔCBD in 1 mM extracellular Ca^{2+}. $Ca_V2.1_{IMAA\text{-}\Delta CBD}$ alone (dMut, ▲) and with co-expression of CaBP1 (dMut+CaBP1, ▲) at 10, 20, and 30 Hz stimulation frequency. Mean EPSP amplitudes were normalized to the first EPSP of train. Data shown are mean ± S.E.M. from 8-14 synaptic pairs. Solid lines are best-fits of a bi-exponential equation as described in Experimental Procedures with the 95% confidence intervals of the fits indicated by dotted lines.

Chapter III

VILIP-2 mediated Ca^{2+}/CaS-dependent regulation of $Ca_V2.1$ channels affects synaptic plasticity

Introduction

In neurons of the central nervous system (CNS), voltage-gated $Ca_V2.1$ channels located in the plasma membrane at the presynaptic terminal open in response to depolarization and allow the influx of Ca^{2+} ions, which triggers neurotransmitter release. Short-term synaptic plasticity causes facilitation and depression of neurotransmitter release during brief trains of stimuli, and this form of synaptic plasticity has been shown to be crucial for encoding information in the nervous system (Zucker and Regehr, 2002). All synapses show some form of short-term facilitation, depression or a combination of both. The extent of facilitation or depression varies at a given synapse, depending on stimulus frequency, neuromodulation and the stage of development (von Gersdorff and Borst, 2002). Additionally, the strength of the synapse depends on the number of vesicles available for release, their Ca^{2+} sensitivity, and on the properties of the Ca^{2+} signals to which the readily releasable pool (RRP) of vesicles responds. The molecular mechanisms leading to synaptic facilitation are poorly understood. The question remains, how is synaptic facilitation mediated? One mechanism of facilitation is well established, as described by the residual Ca^{2+} hypothesis, whereby facilitation is caused by residual Ca^{2+} that remains in the nerve terminal from the Ca^{2+} influx during a previous action potential (AP), leading to an increase in release (Katz and Miledi, 1968; Zucker and Regehr, 2002). However, the molecular

mechanism through which residual Ca^{2+} causes facilitation has been unknown. In sympathetic cervical ganglion (SCG) neurons, it was shown that residual Ca^{2+} in presynaptic terminals can act through neuronal Ca^{2+} sensor proteins (CaS) to cause Ca^{2+}-dependent facilitation (CDF) of $Ca_V2.1$ channels and induce short-term synaptic facilitation (Mochida et al., 2008). At the calyx of Held, $Ca_V2.1$ channels are required for synaptic facilitation (Ishikawa et al., 2005), and a component of short-term facilitation has been correlated with CDF of $Ca_V2.1$ channels (Cuttle et al., 1998; Inchauspe et al., 2004).

In non-neuronal cells, $Ca_V2.1$ channels undergo similar dual feedback regulation by Ca^{2+} that is controlled by CaS protein calmodulin (CaM). CDF and CDI are mediated by binding of Ca^{2+}/CaM to two sites in the C-terminal domain of the $Ca_V2.1$ channel $\alpha1$ subunit composed of an IQ-like motif (IM) and a CaM-binding domain (CBD) (Lee et al., 2000; Lee et al., 2003). Mutation of the IM site (IM-AA) abolishes CDF, whereas deletion of the CBD (△CBD) reduces inactivation and preserves facilitation. SCG neurons transfected with $Ca_V2.1$ channels show short-term synaptic facilitation and depression, and mutations of the IQ-like domain and CBD block synaptic facilitation and depression, respectively (Mochida et al., 2008). Studies have shown that two neuronal CaS proteins, CaBP1 and VILIP-2, bind to shared CaM-regulatory sites on $Ca_V2.1$ channels and modulate their activity (Lee et al., 2002; Few et al. 2005; Lautermilch et al. 2005). While CaBP1 positively shifts the voltage dependence of $Ca_V2.1$ activation, enhances inactivation, and prevents paired-pulse facilitation and CDF during trains of depolarizations of $Ca_V2.1$ channels, VILIP-2 slows the rate of inactivation and reduces inactivation of Ca^{2+} currents during trains of repetitive depolarization (Few et al. 2005; Lautermilch et al. 2005). The differential effects of VILIP-2 and CaBP1 suggest that binding of CaS proteins may play a role in modulation of CDF and

CDI, which may be an important determinant of Ca^{2+} entry in neurotransmission. Thus, CaM and other CaS proteins may respond to residual Ca^{2+} as "facilitation sensors" by binding to the C-terminus of Cav2.1 channels and causing CDF of the presynaptic Ca^{2+} current.

Short-term synaptic plasticity is diverse at different synapses in the central (CNS). Because CaM is ubiquitously expressed, other proteins must determine the diversity of short-term synaptic plasticity. CaM is the best studied CaS protein, it is a multipurpose intracellular modulator, mediating multiple Ca^{2+}-regulated processes. CaM is composed of four functional EF-hand Ca^{2+} binding sites arranged in pairs connected by a flexible α-helical central linker (DeMaria et al., 2001; Lee et al., 2003). Unlike CaM, neuronal CaS proteins contain 4 EF-hands but generally bind 2 or 3 Ca^{2+} ions because one or two of their EF-hands are not functional (Burgoyne and Weiss, 2001; Mikhaylova et al., 2011). They are found mainly in neurons and are thought to be involved in the fine-tuning of calcium-dependent processes in the nervous system. Visinin-like proteins (VILIPs) are members of the neuronal CaS protein superfamily that have been shown to regulate a variety of cellular processes (Braunewell and Klein-Szanto, 2009). VILIP-2 is primarily expressed in the caudate-putamen, neocortex, and hippocampus, including both pyramidal neurons and dentate granule cells (Paterlini et al., 2000). Punctate labeling in the neuropil of the hippocampus and cerebral cortex suggests localization of VILIP-2 in nerve terminals as well (Saitoh et al., 1994, 1995), together with $Ca_V2.1$ channels (Westenbroek et al., 1995).

How VILIP-2 regulation of $Ca_V2.1$ channels contributes to short-term synaptic plasticity is unknown. A recent study has demonstrated that CaBP1 promotes synaptic depression at sympathetic neuron synapses transfected with $Ca_V2.1$ channels by binding to CaS sites on the C-terminus of the channel and modulating Ca^{2+} channel properties (Leal et

al., 2010). Further evidence has implicated the related neuronal calcium sensor-1 (NCS-1) to function as a "facilitation sensor" in synaptic transmission (Tsujimoto et al., 2002; Sippy et al., 2003). In this study, we use a model synapse to express VILIP-2 presynaptically with $Ca_V2.1$ channels to probe the functional properties of VILIP-2 regulation in synaptic transmission. Our results show that VILIP-2 significantly supports synaptic facilitation and is dependent on direct binding to CaS binding sites on the C-terminus of $Ca_V2.1$ channels under conditions of high release probability. These results further define the functional role of CaS protein-dependent modulation of short-term synaptic plasticity. Given the widespread distribution of $Ca_V2.1$ channels throughout the nervous system, the cell type–specific modulation of $Ca_V2.1$ by VILIP-2 may fundamentally determine the action of residual Ca^{2+} signals and the functional output of synaptic activity.

Experimental Methods

Expression of $Ca_V2.1$ in Cultured SCG Neurons

SCG neurons were cultured as described to allow synapse formation (Mochida et al., 1996). cDNAs encoding WT $\alpha_12.1$ subunit, dMut $\alpha_12.1_{IMAA/\Delta CBD}$ containing mutations in the IQ-like and calmodulin-binding domains, sMut $\alpha_12.1_{IMAA}$ containing mutation only in the IQ-like motif, VILIP-2 and eGFP were microinjected into the nuclei of SCG neurons through glass micropipettes with 5% fast-green dye (Sigma-Aldrich, St. Louis, MO). Entry of the constructs into the cell nucleus was monitored by the intensity of green dye in the nucleus. The cells were maintained at 37 ^oC in a 95% air, 5% CO_2-humidified incubator for 48 hours, after which, the injected neurons were identified with an inverted microscope (Olympus) equipped with an epifluorescence unit.

Synaptic Transmission Between SCG Neurons

EPSPs were recorded from SCG neurons cultured for 6 weeks as described (Mochida et al., 1996). Conventional intracellular membrane potential recordings were made from two neighboring neurons using microelectrodes filled with 1 M KAc (70-90 MΩ). EPSPs were recorded from a non-injected neuron while action potentials were generated in the injected, presynaptic neuron expressing the $\alpha_1$2.1 channels and CaS protein by passing 1-2 nA of current for 5 ms through an intracellular recording electrode (Intracellular IE-201, Warner Instruments, Hamden, CT). All endogenous synaptic transmission was blocked by bath application of 3 μM ω-conotoxin GVIA in a modified Krebs' solution consisting of 136 mM NaCl, 5.9 mM KCl, 1 mM $CaCl_2$, 1.2 mM $MgCl_2$, 11 mM glucose, and 3 mM Na-HEPES (pH 7.4). For recording sub-threshold EPSPs, the membrane potential of the postsynaptic cell was held at -70 or -80 mV by passing current (0.2-0.4 nA) through the recording electrode. Electrophysiological data were collected using PULSE (Heka Electronik, Lambrecht, Germany) and analyzed with Igor Pro (Wavemetrics, Lake Oswego, OR). Each protocol was repeated 10 times and averaged for each synapse. The EPSP amplitudes were averaged to account for variation in transmitter release following repetitive action potentials. The peak amplitudes from baseline were averaged and normalized to the first EPSP of each train and plotted against action potential number. Data values with associated error shown in the text and figures represent mean ± S.E.M. Statistical significance for comparisons of the various expression vectors were performed using unpaired Student t-test. Mean data for EPSP trains were fit with bi-exponential functions $C+A\exp(-t/\tau1)+(1-A-C)\exp(-t/\tau2)$. $\tau1$ describing facilitation and the absolute value were poorly defined by the data and fixed at reasonable values for fitting. Best-fit lines (solid) and 95% confidence estimates for these fits (dotted

lines) are provided in the figures.

Patch Clamp Recordings in SCG Neurons

SCG neurons were injected one week after dissociation and plating. cDNAs encoding WT $\alpha_1 2.1$ subunit and eGFP, with or without VILIP-2, were microinjected into the nuclei of SCG neurons through glass micropipettes with 5% fast-green dye (Sigma-Aldrich, St Louis, MO). Entry of the constructs into the cell nucleus was monitored by the intensity of green dye in the nucleus. The cells were maintained at 37 ^oC in a 95% air, 5% CO_2-humidified incubator for 24 hours, after which, the injected neurons were identified with an inverted microscope (Olympus) equipped with an epifluorescence unit. For whole-cell voltage clamp recordings, patch pipettes of borosilicate glass with resistances of 3-6 MΩ were used to record P/Q-type Ca^{2+} currents from injected SCG neurons. Pipettes were filled with intracellular solution containing (in mM) N-methyl-D-glucamine (125), TEA-Cl (10), HEPES (10), $MgCl_2$ (1), EGTA (0.5), MgATP (4), and Na_2GTP (0.3), and Tris-creatine phosphate (14) adjusted to pH 7.2 with methanesulfonic acid. The extracellular solution contained (in mM) TEA-Cl (141.9), $MgCl_2$ (1.2), HEPES (3), and $CaCl_2$ (1) adjusted to pH 7.4 with NaOH. P/Q-type Ca^{2+} currents were isolated by bath application of (in μM) nifedipine (10), SNX-482 (0.1), ω-conotoxin GVIA (3), and TTX (1) to block L-, R-, and N-type Ca^{2+} currents and sodium currents, respectively. Recordings were obtained using an HEKA EPC10 amplifier (HEKA Elektronik, Lambrecht, Germany) with PULSE software and filtered at 5 kHz. All currents were generated by depolarizing from a holding potential of -80 mV. Leak and capacitive transients were subtracted using a P/-4 protocol. Data was analyzed using Igor Pro (Wavemetrics, Lake Oswego, OR). All averaged data and error bars represent the mean ± S.E.M.

Results

VILIP-2 does not affect paired-pulse plasticity in 1mM extracellular Ca^{2+}

Short-term synaptic plasticity is diverse, with facilitation, depression, or facilitation followed by depression observed at different synapses in the central nervous system (Zucker and Regehr, 2002). As CaM is ubiquitously expressed, other proteins must determine the diversity of short-term synaptic plasticity. VILIP-2 reduces inactivation and enhances facilitation of $Ca_V2.1$ channels, whereas related CaS protein CaBP1 enhances inactivation (Lautermilch et al., 2005; Lee et al., 2002). We tested the hypothesis that a small increase in Ca^{2+} influx by VILIP-2 through regulation of $Ca_V2.1$ channels would be sufficient to increase release because of the non-linear relationship between Ca^{2+} influx and neurotransmitter release. We expressed $Ca_V2.1$ channels and VILIP-2 by injection of cDNA into an identified SCG neuron. Synaptic transmission at SCG synapses was measured by using intracellular recordings in transfected cells from WT and WT+VILIP-2 synapses under conditions of low release probability (1 mM extracellular Ca^{2+}). We first looked at the time course of short-term synaptic plasticity by varying the interstimulus interval (ISI). In control WT neurons, a typical facilitation response for SCG neurons was evoked by paired stimulations at 50 ms, in which the second excitatory postsynaptic response (EPSP) was facilitated relative to the first (paired-pulse facilitation (PPF); Figure 3.1*BI*; Mochida et al., 2008). Expression of VILIP-2 did not significantly affect the paired-pulse ratio (PPR; size of the response to the second pulse relative to the first) compared to WT expressing synapses (Figure 3.1*B* and Figure 3.1*C*). Both groups showed PPF at intermediate ISI that decayed to baseline at the longest ISI tested (250 ms). Synapses expressing VILIP-2 showed PPF that was indistinguishable from control WT synapses at peak PPF (WT PPR_{80ms} 1.36 $\pm$ 0.12 (n=23); VILIP-2 PPR_{80ms}, 1.22 $\pm$

0.14 (n=24), $p > 1.0$, unpaired Student's t-test; Figure 3.1*B* and Figure 3.1*C*). Notably, peak PPF for VILIP-2 synapses occurred at the second peak for WT $Ca_V2.1$ expressing synapses. However, the amount of PPF at 80 ms ISI was not significantly different (WT PPR_{80ms} 1.37 $\pm$ 0.12 (n=29); VILIP-2 PPR_{80ms}, 1.59 $\pm$ 0.17 (n=27), $p > 0.05$, unpaired Student's t-test; Figure 3.1*B* and Figure 3.1*C*). Thus, we conclude that VILIP-2 does not affect paired-pulse plasticity. This is in agreement with these findings that VILIP-2 and $Ca_V2.1$ channel coexpression does not alter Ca^{2+} current facilitation measured in paired-pulse experiments in transfected non-neuronal cells (Lautermilch et al., 2005).

We next looked at the basal neurotransmitter release probability by comparing the amplitude of the first EPSP in control synapses to VILIP-2-expressing neurons. A decrease in EPSP initial amplitude has been previously observed to significantly increase PPR, it is therefore possible that VILIP-2 may affect initial release probability but facilitate to the maximal level of endogenous CaM. We detected no obvious changes in the amplitude of the synaptic response between WT and VILIP-2 expressing synapses (WT=2.97 $\pm$ 0.22 mV, n=20; WT+VILIP-2=2.95 $\pm$ 0.31 mV, n=18). Moreover, there was no significant change in the kinetics of the EPSP (Figure 3.1*A*). The lack of effect of VILIP-2 on basal transmission and paired-pulse plasticity were further supported by whole-cell voltage-clamp recordings made from SCG neuronal cell bodies where Ca^{2+} currents were elicited by step-pulse depolarizations (Figure 3.1*D*). The total Ca^{2+} current and peak amplitude of elicited current were similar to those reported previously for transfected $Ca_V2.1$ channels (Mochida et al., 2008). Both WT and VILIP-2 expressing synapses showed similar amount of Ca^{2+} channel expression (Figure 3.1*D*). To confirm the lack of effect of VILIP-2, we next transfected SCG neurons with mutant $Ca_V2.1$ channels lacking CaS binding domains, IM-AA/△CBD.

Mutation of the IQ-like motif (IM-AA) combined with the deletion of the CBD prevents VILIP-2 binding to $Ca_V2.1$ channels (Lautermilch et al., 2005). Co-expression of VILIP-2 had no effect on these double mutant $Ca_V2.1_{IM\text{-}AA/\triangle CBD}$ (dMut) channels in paired-pulse experiments (Figure 3.2*A*-Figure 3.2*C*). $Ca_V2.1_{IM\text{-}AA/\triangle CBD}$ channels showed much less PPF at intermediate ISI compared to WT $Ca_V2.1$ channels (Figure 3.1*B*, black circles), but no difference was observed between WT and VILIP-2 synapses. Evidently, although VILIP-2 modulates inactivation differently from CaM, it can mediate Ca^{2+}-dependent paired-pulse facilitation of Ca^{2+} current (Lautermilch et al., 2005) and PPF similarly. At the same time it does not exclude the possibility that activation of VILIP-2 depends on residual Ca^{2+} during activity-dependent plasticity.

VILIP-2 does not affect activity-dependent plasticity in 1mM external Ca^{2+}

Our previous work has shown that $Ca_V2.1$ channels expressed in SCG neurons demonstrate Ca^{2+}-dependent facilitation (CDF) and Ca^{2+}-dependent inactivation (CDI) of Ca^{2+} currents mediated through CaS proteins that bind the $Ca_V2.1$ subunit carboxyl terminus and induce short-term synaptic facilitation and rapid synaptic depression, respectively (Mochida et al., 2008). VILIP-2 slows the rate of Ca^{2+}-dependent inactivation of $Ca_V2.1$ channels during single long depolarizations and enhances facilitation during extended trains of stimuli in a Ca^{2+}-dependent manner (Lautermilch et al., 2005). Because VILIP-2 has no effect on facilitation by paired-pulses, it is likely that the increase in facilitation during trains of stimuli reflects slowing of cumulative Ca^{2+}-dependent inactivation (Lautermilch et al., 2005). We next investigated whether VILIP-2, which is involved in the activity-dependent facilitation of Ca^{2+} currents, is capable of modifying synaptic facilitation during trains of

activity. To investigate the potential effect of VILIP-2 on $Ca_V2.1$ channels during trains of activity, we stimulated synapses at varying frequencies and recorded EPSPs during each stimulation in the presence of 1 mM extracellular Ca^{2+} to give a low probability of neurotransmitter release (Figure 3.3*A* and Figure 3.3*B*). In control synapses expressing WT $Ca_V2.1$ channels alone, we observed synaptic facilitation that decayed in a pulse-wise manner at all stimulus frequencies (Figure 3.3*B*). In order to include all of the data points in our statistical analysis, the mean synaptic responses were fit to a bi-exponential function, the time constant for depression was derived from this fit, and the value for maximum facilitation was estimated by extrapolation to time of the second stimulus in the train. Errors in the fits and parameter estimates were expressed as 95% confidence limits (Figure 3.3*B*, dotted lines). This profile of synaptic facilitation and depression resembles regulation of $Ca_V2.1$ channels by endogenous CaM. Surprisingly, synapses expressing VILIP-2 showed synaptic facilitation followed by synaptic depression that was similar to WT synapses (Figure 3.3*C*). At 30 Hz, no difference in the rate of facilitation or depression was observed (Figure 3.3*C*). These results illustrate that effects of short-term synaptic plasticity are not always accurately extrapolated from results in non-neuronal cells. VILIP-2 was unable to increase facilitation of synaptic transmission in paired pulses or trains of stimuli under conditions of low release probability (Figures 3.1 and Figure 3.3). As a further control for the specificity of VILIP-2, we tested VILIP-2 on SCG neurons expressing the $Ca_V2.1_{IM\text{-}AA/\triangle CBD}$ mutant channel. As expected from the results of Figures 3.1 and 3.3, co-expression of VILIP-2 did not significantly increase facilitation during trains of stimuli at 10, 20, or 30 Hz (Figure 3.4A and Figure 3.4B). These results are consistent with the conclusion that the $Ca_V2.1_{IM\text{-}AA/\triangle CBD}$ mutation completely prevents CaS protein regulation. Evidently, although VILIP-2 enhances

facilitation of $Ca_V2.1$ channels during extended trains of stimuli in transfected non-neuronal cells (Lautermilch et al., 2005), it does not affect synaptic transmission in SCG neurons under these conditions, which may result from the differences in Ca^{2+} dynamics in presynaptic active zones versus non-neuronal cells.

VILIP-2 Enhances Synaptic Facilitation in 2 mM external Ca^{2+}

Facilitation during trains of stimuli in SCG neuron synapses is robust, approaching 2.22-fold at 30 Hz in 1 mM extracellular Ca^{2+} (Figure 3.3*B*). This substantial level of facilitation may represent the maximum possible at these synapses. We hypothesized that this high level of basal synaptic facilitation may occlude effects of VILIP-2. Therefore, we raised external Ca^{2+} from 1 mM to 2 mM in order to enhance release probability and increase synaptic depression. Under these conditions, control synapses show rapid synaptic depression at all three stimulus frequencies (Figure 3.4*A* and Figure 3.5*B*), but synapses expressing VILIP-2 show significantly more facilitation and significantly less depression at 20 Hz and 30 Hz than controls (Figure 3.5*B*, $p < 0.05$). At 30 Hz, VILIP-2 restores synaptic facilitation during the first five pulses followed by pulse-wise synaptic depression (1.37 ± 0.08, $p < 0.01$; Figure 3.5*B*). Despite this restoration of facilitations, peak synaptic facilitation observed with VILIP-2 in 2 mM Ca^{2+} (Figure 3.5*B*) was 27% less than the corresponding control without VILIP-2 in 1 mM extracellular Ca^{2+} (Figure 3.3*B*, $p < 0.05$). The increase in facilitation during stimulus trains in 2 mM Ca^{2+} relative to controls highlights the ability of VILIP-2 to facilitate neurotransmitter release at high frequencies of stimulation when extracellular Ca^{2+} is elevated and basal neurotransmitter release is high. The synaptic facilitation during trains of stimuli indicates that VILIP-2 binding to $Ca_V2.1$ channels slows their cumulative Ca^{2+}-

dependent inactivation and thereby enhances their Ca^{2+}-dependent facilitation (Lautermilch et al., 2005). Facilitation of EPSPs by VILIP-2 in the depressed state of the synapse could transiently compensate for the effect of depression, thereby stabilizing the neurotransmitter during a train of activity.

CaS Binding Sites of Ca_V2.1 Channels Are Essential for VILIP-2 Modulation

To confirm that the effect of VILIP-2 in 2mM Ca^{2+} is due to direct interaction with the CaM binding sites on the channel, we co-expressed VILIP-2 with double mutant $Ca_V2.1_{IM\text{-}AA/\triangle CBD}$ (dMut) channels. Under these conditions of high release probability in 2 mM Ca^{2+}, dMut $Ca_V2.1_{IM\text{-}AA/\triangle CBD}$ channels undergo rapid synaptic depression, as seen at other synapses when external Ca^{2+} is elevated (Figure 3.6*A* and Figure 3.6*B*; Zucker and Regehr, 2002). Co-expression of VILIP-2 has no effect on depression or facilitation of dMut $Ca_V2.1_{IM\text{-}AA/\triangle CBD}$ channels at all frequencies tested (Figure 3.6*B*). This result is consistent with the conclusion that VILIP-2 prevents rapid Ca^{2+}-dependent channel inactivation and enhances Ca^{2+}-dependent facilitation when external Ca^{2+} and basal release probability are high by interaction with the CaS protein regulatory site on Ca_V2.1 channels. Evidently, when bound in place of CaM, VILIP-2 can switch SCG synapses from synaptic depression to facilitation.

Single CaS binding site is sufficient for VILIP-2 regulation of Ca_V2.1 channels

It has previously been shown that binding of CaM to the IQ-like motif, IM, induces Ca^{2+}-dependent facilitation (Lee et al., 2003; Liang et al., 2001). Furthermore, studies have shown that VILIP-2 also binds to the IM domain and that mutation of its first two residues

(IM-AA) completely prevents enhancement of facilitation by VILIP-2 (Nanou et al., 2012). Although mutation of both CaS sites abolished VILIP-2 facilitation, these results suggest that the IM site is necessary and sufficient for VILIP-2 modulation of Ca^{2+}-dependent facilitation. Therefore, we tested whether mutation of the first two residues (IM-AA) of the IQ-like motif of $Ca_V2.1$ channels can prevent VILIP-2 mediated synaptic facilitation during high release probability in paired-pulse plasticity. Intracellular recordings from pairs of connected neurons expressed with WT $Ca_V2.1$ channels alone exhibited robust paired-pulse depression (PPD) of EPSPs at short and intermediate intervals (Figure 3.7*A* and Figure 3.7*B*). In WT synapses, the average PPR for 50 ms was 0.60 $\pm$ 0.12 (n=10; Figure 3.7*B*). In contrast, expression of VILIP-2 increases PPF significantly at 50 ms and preserves low level PPF at intervals less than 150 ms (Figure 3.7*A* and Figure 3.7*B*). Synapses expressing VILIP-2 showed PPF that peaked at 50 ms and was markedly different from control synapses (WT+VILIP-2 PPR_{50ms}, 1.25 $\pm$ 0.13 (n=10), *** $p < 0.001$ compared to WT synapses, unpaired Student's t-test; Figure 3.7*B*). As ISI was extended, PPF of VILIP-2 synapses decayed faster than WT synapses. We next transfected SCG neurons with mutant $Ca_V2.1$ channels lacking only one CaS binding domain, IM-AA. Expression of VILIP-2 with mutant $Ca_V2.1_{IM\text{-}AA}$ (sMut) channels in paired-pulse experiments prevented VILIP-2 mediated PPF (Figure 3.7*A*-Figure 3.7*B*). Single mutant $Ca_V2.1_{IM\text{-}AA}$ channels showed PPD at short and intermediate ISI compared to VILIP-2 expressing synapses (sMut PPR_{50ms} 0.72 $\pm$ 0.06 (n=9), VILIP-2 PPR_{50ms}, 1.25 $\pm$ 0.13 (n=10); sMut PPR_{80ms} 0.78 $\pm$ 0.12 (n=9) ; VILIP-2 PPR_{80ms} 1.07 $\pm$ 0.11 (n=10), Figure 3.7*B*), but little difference was observed between WT and sMut $Ca_V2.1_{IM\text{-}AA}$+VILIP-2 synapses. Our results indicate that the IM site is necessary for VILIP-2 mediated facilitation and mutation of the IM site completely prevents synaptic facilitation by

VILIP-2 in paired-pulses. Overall, these results suggest that synaptic facilitation requires high affinity binding of VILIP-2 to the IM site, which enhances facilitation in $Ca_V2.1$ channels during conditions of high external Ca^{2+}.

Discussion

The results presented in this chapter show that Ca^{2+}-dependent facilitation of synaptic transmission requires high-affinity binding of VILIP-2 to the IM site and CBD, which induces a conformational change that enhances facilitation in $Ca_V2.1$ channels when $[Ca^{2+}]_o$ is elevated. VILIP-2 slows the rate of synaptic depression during trains of activity in a pulse-wise manner by slowing the rate of inactivation of $Ca_V2.1$ channels present at the terminal (Lautermilch et al., 2005; Nanou et al., 2012). Thus, in normal 1mM extracellular Ca^{2+}, expression of VILIP-2 can provide bidirectional control of synaptic facilitation and depression.

Short-term synaptic facilitation has been attributed to the lasting effect of Ca^{2+} in the presynaptic terminal (Zucker and Regehr, 2002). However, the molecular mechanism by which "residual calcium" acts to increase the amount of neurotransmitter during successive action potentials in not known. Two hypotheses have been proposed to account for short-term facilitation, "facilitation sensor" and "buffer saturation". Both these hypotheses propose residual Ca^{2+} does not act directly on the Ca^{2+} sensor for neurotransmitter release but rather on other proteins capable of sensing Ca^{2+}. The "buffer saturation" hypothesis proposes that facilitation can occur as a result of progressive and local saturation of fast endogenous Ca^{2+} buffers, like calbindin, in the presynaptic terminal during a train of action potentials. As a result, there is a gradual increase of intracellular free Ca^{2+} concentration due to gradual reduction of buffering capacity resulting in synaptic facilitation (Blatow et al., 2003; Rozov

et al., 2001). In contrast, the "facilitation sensor" hypothesis proposes that residual Ca^{2+} binds to a Ca^{2+} sensor other than that for neurotransmitter release to increase the probability of release (Sippy et al., 2003; Tsujimoto et al., 2002; Mochida et al., 2008). Activation of this "facilitation sensor" may increase Ca^{2+} entry and thereby enhance neurotransmitter release according to the power law or may directly modulate the vesicular release machinery to enhance neurotransmitter release. Collectively, our data support the notion that selective Ca^{2+}-dependent regulation of presynaptic Ca^{2+} channels underlies several key aspects of short-term plasticity by facilitation sensor proteins.

At the calyx of Held and hippocampal synapses, Ca^{2+}-dependent facilitation of presynaptic $Ca_V2.1$ channels by a related neuronal CaS protein, NCS-1, leads to synaptic facilitation (Tsujimoto et al., 2002; Sippy et al., 2003). These studies argue that residual Ca^{2+} acts on a facilitation sensor, like NCS-1 and VILIP-2, which causes Ca^{2+}-dependent facilitation of $Ca_V2.1$ channels and therefore enhances synaptic facilitation. However, the site of NCS-1 action has not been identified so the exact molecular steps involved remain unknown. NCS-1 and VILIP-2 have similar sequence homology (Haeseleer et al., 2000; Lautermilch et al., 2005), so we can speculate that NCS-1 responds to residual Ca^{2+} and binds to CaS sites on $Ca_V2.1$ channels to cause activity dependent increase in presynaptic Ca^{2+} transients.

CaM can mediate both Ca^{2+}- dependent facilitation and Ca^{2+}-inactivation of $Ca_V2.1$ channels in heterologous expression systems (Lee et al., 2002; Lee et al., 2003; Lautermilch, et al., 2005). SCG synapses transfected with $Ca_V2.1$ channels show short-term synaptic facilitation and depression, and mutations of the IM site and CBD block synaptic facilitation and depression, respectively (Mochida et al., 2008). Because CaM is ubiquitously expressed,

other CaS proteins may provide diversity in translating Ca^{2+} influx signals in neurons. CaS proteins transmit the Ca^{2+} signals to other cellular components. Ca^{2+} induces a conformational change resulting in the exposure of hydrophobic surface(s), thereby allowing interaction with other target proteins (Burgoyne and Weiss, 2001; LaPorte et al., 1980). Like CaM, CaBP1 has been shown to bind to $Ca_V2.1$ channels and produce inactivation resulting in reduced Ca^{2+} entry during trains of activity, which blocks synaptic facilitation and contributes to depression (See Chapter I; Leal et al., 2010). These considerations suggest that VILIP-2 may not be the only neuronal CaS protein capable of functioning as a CaS for synaptic plasticity to affect facilitation or depression. The possibility emerges that differences in levels of endogenous CaS proteins might underlie differences in synaptic facilitation and depression among different synaptic terminals. Our data and those of others support the hypothesis that residual Ca^{2+} acts on a facilitation sensor, like VILIP-2, to facilitate synaptic responses by increasing CDF of $Ca_V2.1$ channels.

In contrast, Ca^{2+} buffer proteins in the cytosol (for example, parvalbumin, and calbindin-D28k) help to stabilize the intracellular Ca^{2+} concentration, allowing proper Ca^{2+} signal generation, and protecting cells from a Ca^{2+} overload (Baimbridge, 1992). Binding of Ca^{2+} to these proteins is characterized by a slow kinetics and a relatively high affinity (and capacity). Upon calcium binding they generally show no exposure of hydrophobic surfaces, and do not interact with target proteins. Our data support the notion that VILIP-2 acts as a facilitation sensor because coexpression of $Ca_V2.1$ channels with mutated IM domain and deletion of the CBD abolish VILIP-2 mediated synaptic facilitation in 2 mM external Ca^{2+}. It is likely, therefore, that other neuronal CaS proteins are involved in intracellular signaling

processes through regulation of a variety of target proteins may modulate synaptic plasticity (Mikhaylova et al., 2011).

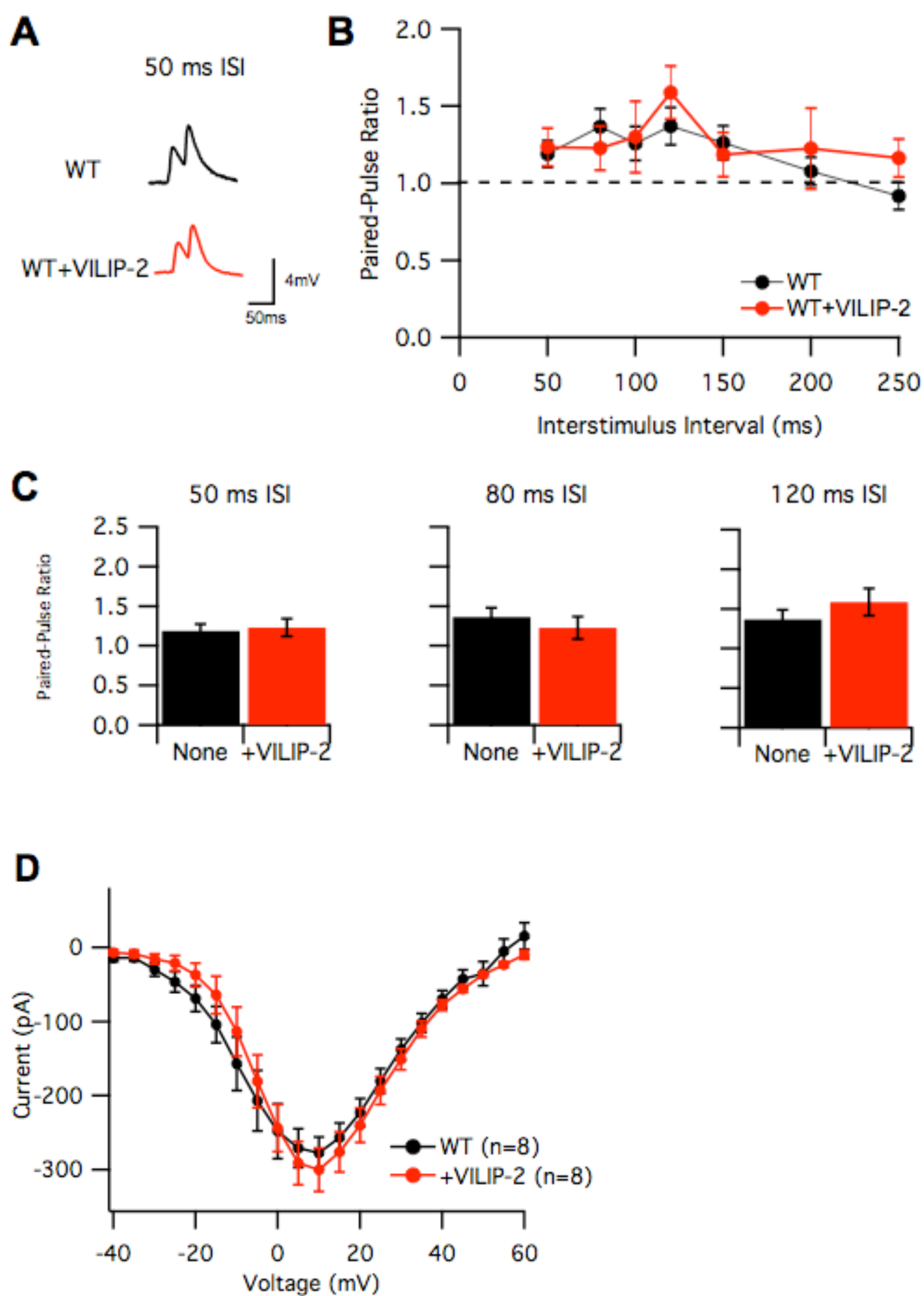
Identification of VILIP-2 as a CaS for facilitation in SCG neurons raises a number of important questions about the functional role of VILIP-1 and VILIP-3 in synaptic transmission. The subfamily of VILIP proteins share 89%-98% sequence homology with each other raising the possibility that VILIP-1 and VILIP-3 can substitute for VILIP-2 in neurons (Braunewell and Klein-Szanto, 2009). VILIP-2 is specifically expressed in the brain (cortex, hippocampus, hypothalamus, midbrain, olfactory bulb) and is noticeable absent in the cerebellum and pons (Kajimoto et al., 1993; Paterlini et al., 2000). The highest amount of VILIP-2 mRNA is observed in the hippocampus (Saitoh et al., 2001; Saitoh et al., 1994). In contrast, VILIP-3 is primarily expressed in the cerebellum; it is also found in the pons and medulla oblongata, and hippocampus, but not in other regions of the brain (Kajimoto et al., 1993; Saitoh et al., 1994). Differential expression of these VILIP isoforms may fine-tune synaptic plasticity in different brain regions. Additionally, differences in Ca^{2+} affinity may distinguish which CaS proteins respond to changes in intracellular Ca^{2+} at the presynaptic terminal (Burgoyne, 2007).

Furthermore, different structural components add to the diversity of CaS sensor proteins' in regulation of target proteins. For example, chimeric studies have shown that differential modulation of VILIP-2 and CaBP1 of $Ca_V2.1$ channels is dependent on N-terminal myristoylation (Lautermilch et al. 2005; Few et al., 2005; Few et al., 2012; Nanou et al., 2012). Removal of the N-terminal myristoyl site from VILIP-2 and CaBP1 reproduces regulation by CaM (Lautermilch et al. 2005; Few et al., 2005; Few et al., 2012; Nanou et al., 2012). Because CaM is present at high concentrations in all cells, it is likely that the neuronal

CaS proteins, which have higher affinity Ca^{2+} binding and are differentially expressed throughout the brain play an important role in shaping the diversity of synaptic transmission in response to activity-dependent changes. In conclusion, multiple mechanisms are involved in synaptic facilitation and depression; the precise constellation depends on the exact circumstances at a given synapse with some mechanisms contributing more than others or not at all. The diversity in CaS proteins adds to a large range of mechanisms that different synapses may tap into in response to different stimulus frequencies to modulate their degree of short-term plasticity.

Figure 3.1. VILIP does not affect paired-pulse plasticity

(A) Representative EPSPs evoked by paired action potentials with 50-ms inter-stimulus interval in the presynaptic neurons expressing $Ca_V2.1$ alone (WT) and co-transfected with CaS protein VILIP-2 (WT+VILIP-2) in the presence of ω-conotoxin GVIA (3 μM). **(B)** Average paired-pulse ratio (PPR) plotted against inter-stimulus interval (ISI) for WT (●) and WT+VILIP2 (●) in 1 mM extracellular Ca^{2+}. PPR equals the amplitude of the second EPSP divided by the amplitude of the first EPSP. **(C)** Paired-pulse ratios for 50 ms, 80 ms, and 120 ms inter-stimulus intervals for WT (black) and WT+VILIP-2 (red). Averaged amplitudes (mean $\pm$ S.E.M.) of the first EPSP recorded for 50-ms ISI: WT, 2.97 ± 0.22 mV (n=20); WT±VILIP-2, 2.95 ± 0.31 mV (n=18; p>0.1 vs. WT). **(A-C)** Data shown are mean ± S.E.M. from 10-20 synaptic pairs. **(D)** $Ca_V2.1$ channel Ca^{2+} currents in SCG neuronal cell bodies. CaS protein expression does not alter total Ca^{2+} channel current in transfected SCG soma. Current-voltage curves generated by eliciting P/Q-type Ca^{2+} currents with a series of depolarizing voltage-steps increasing by 5 mV increments for WT (●, n=8) and WT+VILIP-2 (●, n=8) in 1mM Ca^{2+} external solution.


A
50 ms ISI
WT
WT+VILIP-2
4mV
50ms
B
Paired-Pulse Ratio
2.0
1.5
1.0
0.5
0.0
0
50
100
150
200
250
Interstimulus Interval (ms)
WT
WT+VILIP-2
C
50 ms ISI
80 ms ISI
120 ms ISI
Paired-Pulse Ratio
2.5
2.0
1.5
1.0
0.5
0.0
None
+VILIP-2
D
Current (pA)
0
-100
-200
-300
-40
-20
0
20
40
60
Voltage (mV)
WT (n=8)
+VILIP-2 (n=8)

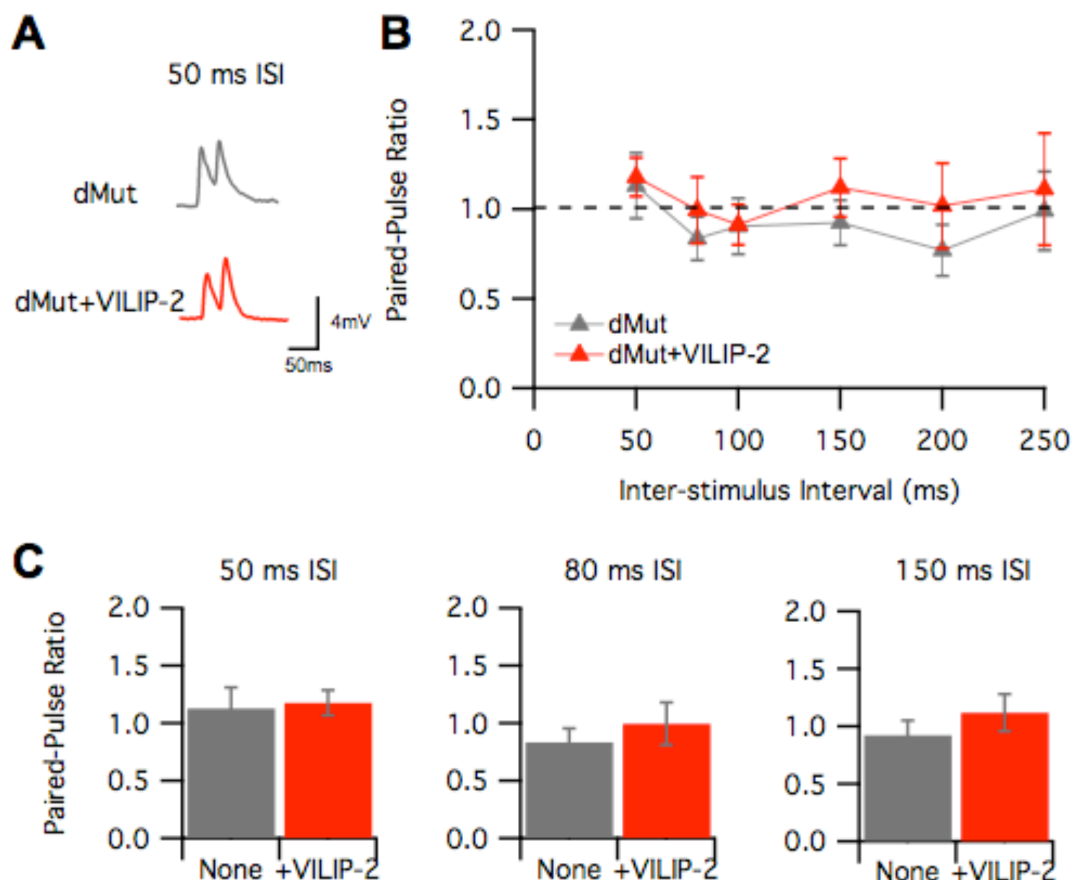


Figure 3.2. VILIP-2 does not alter CaS-dependent plasticity

(A) Averaged EPSP traces (10 traces) elicited by neurons expressing $Ca_V2.1_{IMAA\text{-}\Delta CBD}$ alone (dMut), or with CaS protein VILIP-2 (dMut+VILIP-2) evoked by paired-action potentials with 50 ms inter-stimulus interval in the presynaptic neurons. (B) Paired-pulse ratio (PPR) plotted against inter-stimulus interval (ISI) for $Ca_V2.1_{IMAA\text{-}\Delta CBD}$ alone (dMut; ▲), dMut+VILIP2 (▲). PPR equals the amplitude of the second EPSP divided by the amplitude of the first EPSP. (C) Paired-pulse ratios for 50, 80, and 150 ms inter-stimulus intervals for $Ca_V2.1_{IMAA\text{-}\Delta CBD}$ alone (dMut; grey) and dMut+VILIP-2 (red). Averaged first EPSP amplitude recorded for 50 ms ISI, dMut=6.6$\pm$1.6 (n=16), dMut+VILIP2 = 4.78$\pm$0.96 (n=13). (A-C) Data shown are mean $\pm$ S.E.M. from 10-15 synaptic pairs.

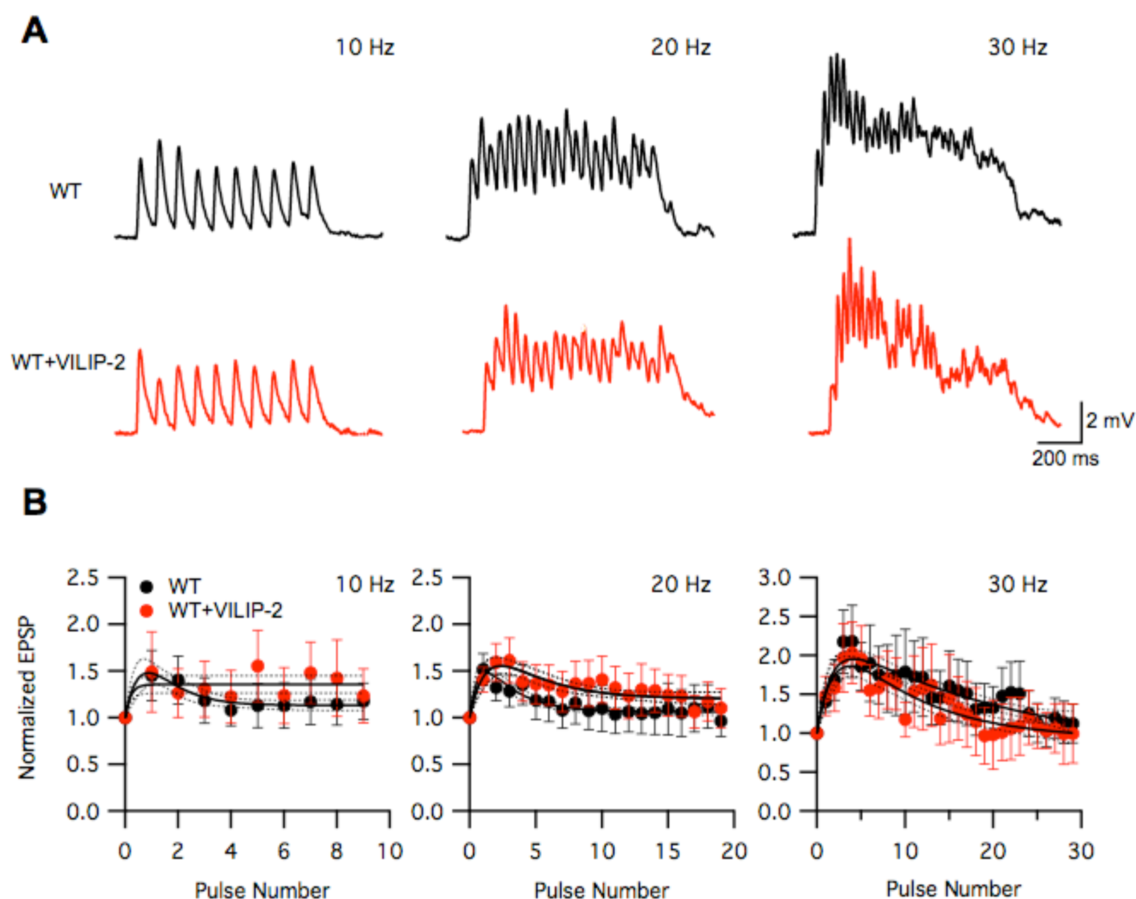


Figure 3.3. VILIP-2 does not affect synaptic facilitation during trains of stimuli in 1mM extracellular Ca^{2+}

(A) Representative EPSPs in 1 mM extracellular Ca^{2+} evoked by repetitive action potentials at 10, 20, and 30 Hz for 1s in the presynaptic neurons expressing wild-type $Ca_V2.1$ alone (WT, black), co-transfected with VILIP-2 (WT+VILIP-2, red). Data from 10 sweeps repeated every 30s at each frequency were averaged. **(B-C)** Mean normalized EPSP amplitude from 10-16 synaptic pairs at 10, 20, and 30 Hz frequency (5 Hz not shown). EPSP amplitudes were normalized to the first EPSP of each train and plotted against action potential number for WT (●) and WT+VILIP-2 (●). Points represent the mean ± S.E.M. Solid lines are best-fits of a bi-exponential equation as described in Experimental Procedures with the 95% confidence intervals of the fits indicated by dotted lines.

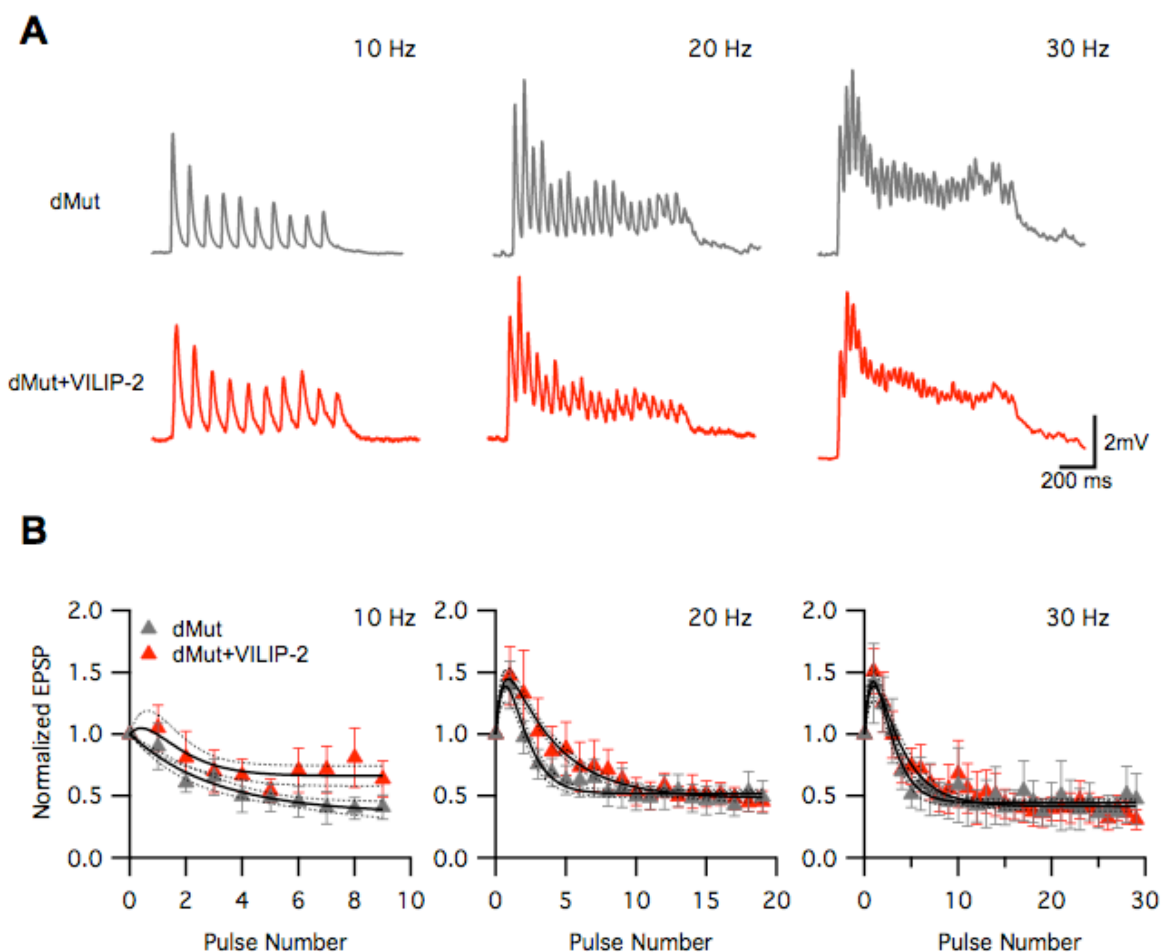


Figure 3.4. Coexpression of VILIP-2 with mutant $Ca_V2.1$ channels shows no change in short-term synaptic plasticity

(A) Representative EPSPs in 1 mM extracellular Ca^{2+} evoked by repetitive action potentials at 10, 20, and 30 Hz for 1s in the presynaptic neurons expressing $Ca_V2.1_{IMAA\text{-}\Delta CBD}$ alone (dMut, grey) or co-transfected with VILIP-2 (dMut+VILIP-2, red). Data from 10 sweeps repeated every 30s at each frequency were averaged. **(B)** Normalized EPSP amplitudes for $Ca_V2.1_{IMAA\text{-}\Delta CBD}$ alone (dMut, ▲) and with co-expression of VILIP-2 (dMut+VILIP-2, ▲) at 10, 20, and 30 Hz stimulation frequency. Mean EPSP amplitudes were normalized to the first EPSP of train. Data shown are mean ± S.E.M. from 8-14 synaptic pairs.

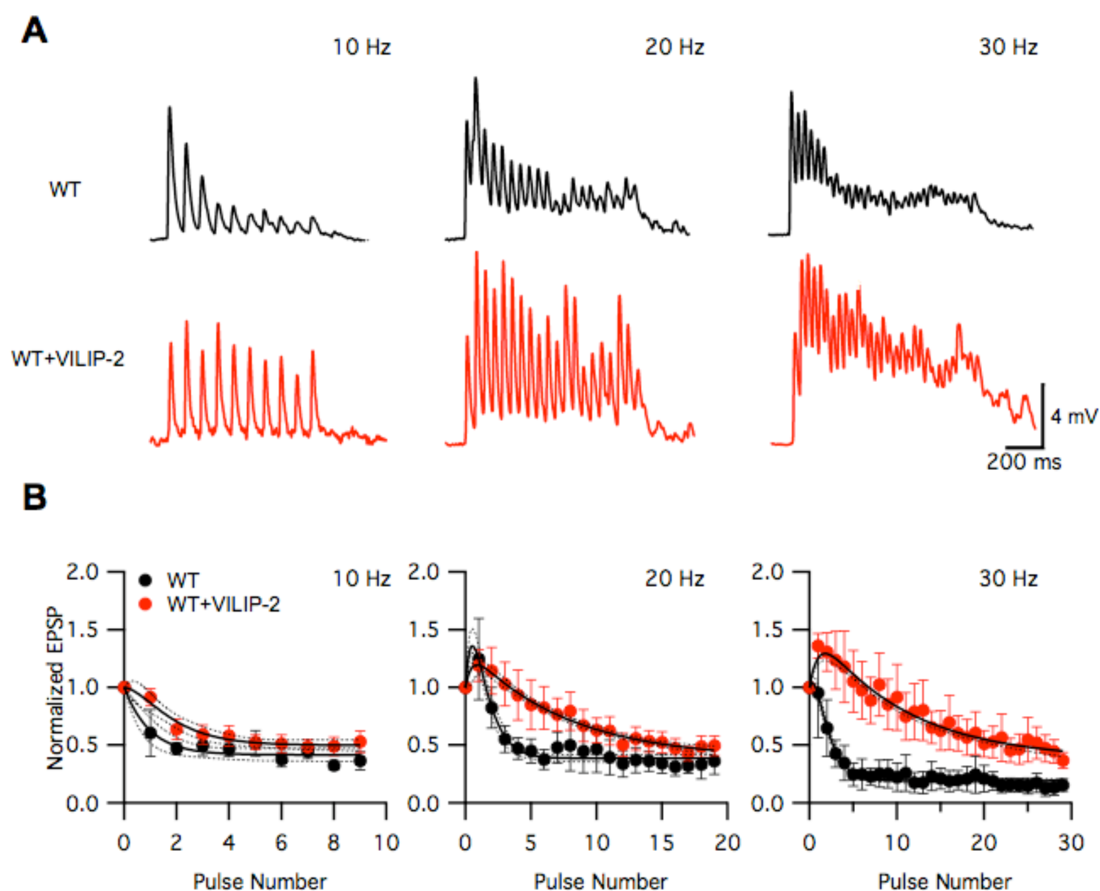


Figure 3.5. VILIP-2 sustains synaptic facilitation in 2mM extracellular Ca^{2+}

(A) Representative EPSPs in 2 mM extracellular Ca^{2+} evoked by repetitive action potentials at 10, 20, and 30 Hz for 1s in the presynaptic neurons expressing wild-type channels (WT, black) or co-transfected with VILIP-2 (WT+VILIP-2, red). Data from 10 sweeps repeated every 30s at each frequency were averaged. **(B)** Normalized EPSPs recorded after expression of $Ca_V2.1$ channels alone WT (●) and with VILIP-2 (●) in 2 mM extracellular Ca^{2+} during trains of action potentials at 10, 20, and 30 Hz.

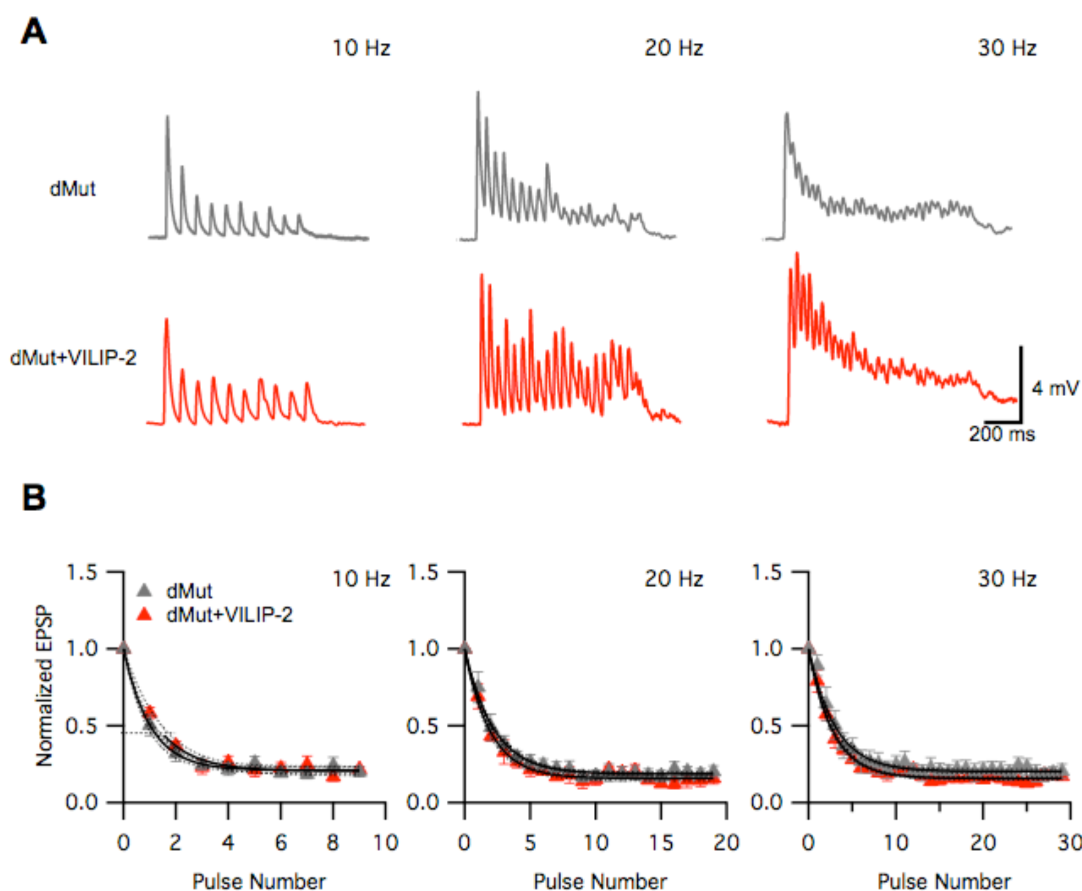


Figure 3.6. VILIP-2 Enhances Synaptic Facilitation via the CaS Protein Binding Site on $Ca_V2.1$ Channels

(A) Representative EPSPs in 2 mM extracellular Ca^{2+} evoked by repetitive action potentials at 10, 20, and 30 Hz for 1s in the presynaptic neurons expressing $Ca_V2.1_{IMAA\text{-}\Delta CBD}$ alone (dMut, grey) or co-transfected with VILIP-2 (dMut+VILIP-2, red). Data from 10 sweeps repeated every 30s at each frequency were averaged. **(B)** Normalized EPSPs recorded after expression of $Ca_V2.1$ channel double mutant $Ca_V2.1_{IMAA\text{-}\Delta CBD}$ alone (dMut, ▲) and with VILIP-2 (dMut+VILIP-2, ▲) in 2 mM extracellular Ca^{2+} during trains of action potentials at10, 20, and 30 Hz. Averaged first EPSP amplitude recorded in 2 mM extracellular Ca^{2+}: WT, 6.99 ± 1.9 mV (n=10); WT+VILIP-2, 5.60 ± 0.98 mV (n=14), dMut, 7.69 ± 1.01 mV (n=8), dMut+VILIP-2, 6.77±1.19 (n=12). Data shown are mean ± S.E.M. from 5-14 synaptic pairs.

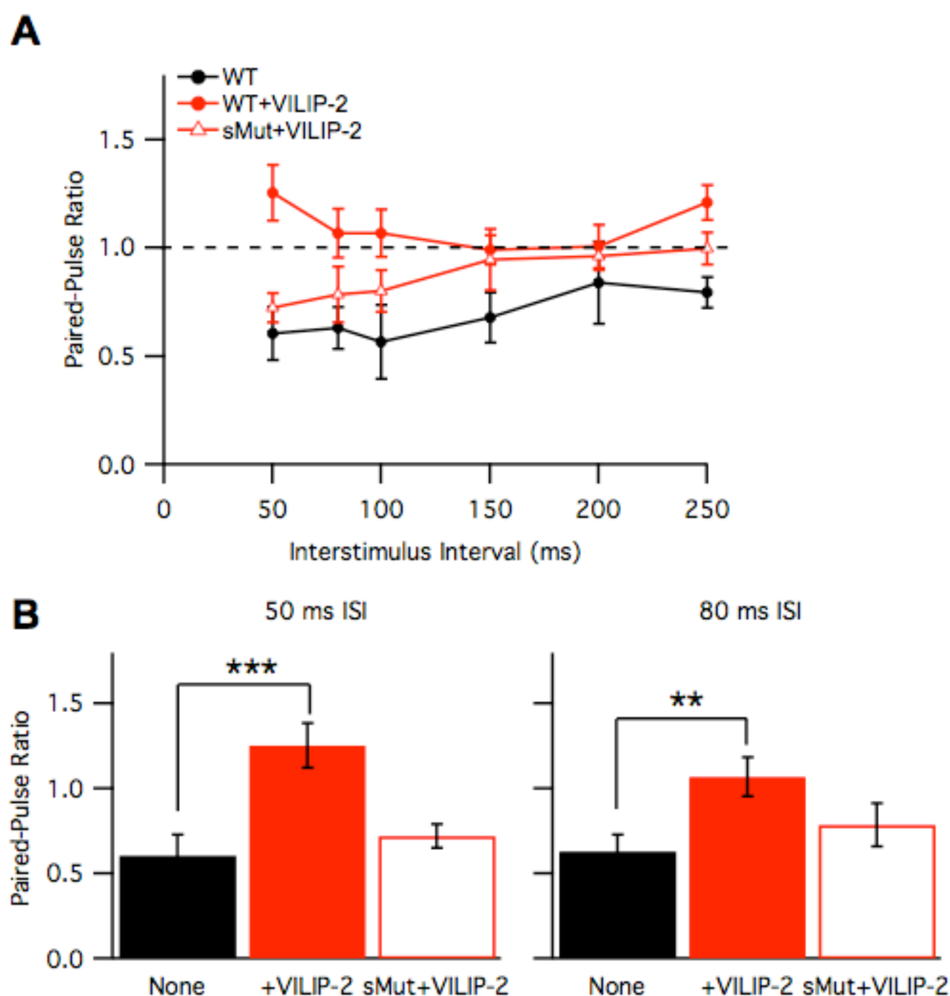


Figure 3.7. IM-AA mutation is sufficient to abolish VILIP-2 mediated synaptic facilitation.

(A) Average paired-pulse ratio (PPR) plotted against inter-stimulus interval (ISI) for WT (●); WT+VILIP2 (●); and IMAA mutant channel, sMut+VILIP-2 (△) in 2 mM extracellular Ca^{2+}. PPR equals the amplitude of the second EPSP divided by the amplitude of the first EPSP. **(B)** Paired-pulse ratios for 50 ms and 80 ms inter-stimulus intervals for WT (black), WT+VILIP-2 (red), sMut+VILIP-2 (open red bar). ** $p < 0.01$, *** $p < 0.001$ unpaired Students t-test for differences compared to WT group. Data shown are mean ± S.E.M. from 9-11 synaptic pairs.

Chapter IV

Conclusions & Discussion

Short-term synaptic plasticity converts the information encoded in the frequency and pattern of action potential firing in the presynaptic terminal into an analog signal for transmission to the postsynaptic neuron. Our results show that this information processing can be controlled in a bi-directional manner at the level of the presynaptic Ca^{2+} channel by CaS proteins, which are poised to modulate these channels and alter neurotransmitter release. Direct regulation of presynaptic $Ca_V2.1$ channels by the CaS proteins CaBP1 and VILIP-2 may serve as a bidirectional switch to control the input-output relationships of synapses in response to trains of action potentials. This conversion of synapses from depressing to facilitating and vice-versa would be expected to have profound consequences for the encoding properties of neural circuits, thereby fine-tuning the synaptic plasticity of different types of synapses (Abbott and Regehr, 2004).

Molecular Analysis of Synaptic Plasticity in SCG Neurons

Analysis of the functional effects of presynaptic Ca^{2+} channel regulation in synaptic transmission is challenging because of the need to alter regulation only in the presynaptic cell. If both presynaptic and postsynaptic cells are transfected, it is not possible to determine whether the observed actions of the transfected proteins on synaptic transmission are caused by presynaptic or postsynaptic effects. Our experiments took advantage of the unique characteristics of SCG neurons as an expression system for studies of presynaptic effects on

synaptic transmission. These neurons lack known CaS proteins, which are specifically expressed in the central nervous system and retina (Burgoyne, 2007; Haeseleer et al., 2002), and they lack $Ca_V2.1$ channels, which are expressed primarily in central neurons (Uchitel et al., 1992; Westenbroek et al., 1995; Wheeler et al., 1994). Thus, SCG neurons provide a null genetic background for studies of these key components of central synapses. The large cell bodies of cultured SCG neurons allow microinjection of cDNA encoding $Ca_V2.1$ channels into individual cell nuclei without damage, which gives expression only in presynaptic cells, and the level of functional expression is in the same range as the endogenous $Ca_V2.2$ channels, which can be specifically and completely blocked by pharmacologically ω-conotoxin GVIA (Mochida et al., 2003). As a result, genetic manipulations are made with relative ease compared to accessing single central nerve terminals. We have overcome technical hurdles to isolate and study the influence of presynaptic $Ca_V2.1$ channel regulation on synaptic efficacy. Like central neurons, synaptic facilitation in SCG neurons depends upon expression of $Ca_V2.1$ channels (Ishikawa et al., 2005; Mochida et al., 2008). In response to activity-dependent stimulation at frequencies 10 Hz to 30 Hz $Ca_V2.1$ channels induce synaptic facilitation followed by depression (Mochida et al., 2008). Both facilitation and the rapid phase of depression are dependent on regulation of $Ca_V2.1$ channels by CaM binding to the IQ and CBD motifs in the C-terminal domain (Mochida et al., 2008). Our unique and powerful expression system has allowed us to directly test the role of CaS proteins, CaBP1 and VILIP-2, in modulation of synaptic plasticity that is dependent on $Ca_V2.1$ channels, and to demonstrate the requirement for CaS protein binding to the IM and CBD motifs using mutant $Ca_V2.1$ channel constructs. By using SCG neurons, we can

attribute synaptic changes produced by expression of CaS proteins, to presynaptic and not postsynaptic mechanisms.

CaS Proteins Override CaM-dependent Synaptic Plasticity in a Ca^{2+}-dependent Manner

Similar to CaM, the CaS proteins are expressed in high levels in central neurons (Burgoyne, 2007; Haeseleer et al., 2000; Paterlini et al., 2000), so it is likely that the levels achieved by exogenous expression in SCG neurons are lower than the levels in their native cell types. Therefore, it was not at all certain that expression of CaS proteins would be able to override the effects of endogenous CaM on synaptic plasticity in SCG neurons. Surprisingly, our results show that specific expression of CaBP1 in presynaptic neurons can indeed override the effects of endogenous CaM. CaBP1 expression blocks Ca^{2+}/CaS-dependent facilitation and enhances rapid synaptic depression when studied under conditions of low release probability in the presence of 1 mM extracellular Ca^{2+}, where synaptic facilitation is prominent. In contrast, CaBP1 did not have detectable effects on synaptic plasticity at 2 mM Ca^{2+}, where release probability is high and synaptic depression is rapid (data not shown). Thus, CaBP1 is able to compete with CaM for binding to their common regulatory site and induce changes in short-term synaptic plasticity under physiological conditions, depending on the level of Ca^{2+} entry and the resulting level of release probability (Chapter I).

In contrast to CaBP1, VILIP-2 has no effect on synaptic plasticity in SCG neurons at 1 mM extracellular Ca^{2+}, but it reduces synaptic depression and enhances synaptic facilitation at 2 mM Ca^{2+}, where synaptic depression is dominant. These results show that VILIP-2 can also override the synaptic plasticity induced by CaM under appropriate conditions of Ca^{2+}

entry. It is noteworthy that the Ca^{2+} dependence of CaBP1 and VILIP-2 effects is opposite, with CaBP1 more effective at low extracellular Ca^{2+} and VILIP-2 more effective at higher extracellular Ca^{2+}. The effects of both of these CaS proteins on synaptic plasticity depend crucially on the form and frequency of Ca^{2+} transients at the presynaptic active zone. Their effects are insignificant at 5 Hz (unpublished results), detectable at 10 Hz and 20 Hz, and greater during 30-Hz trains of stimuli in 1-2 mM extracellular Ca^{2+}. Analysis of the effects of regulatory proteins on synaptic plasticity requires direct measurements of synaptic function where the unique characteristics of active zones define the mode, extent, and Ca^{2+} dependence of regulation.

Regulation of Synaptic Plasticity by CaS Proteins Is Bidirectional

At 1 mM extracellular Ca^{2+}, CaBP1 is effective in reducing synaptic facilitation and enhancing synaptic depression, whereas VILIP-2 has no effect. However, when extracellular Ca^{2+} was increased to the more physiological level of 2 mM, VILIP-2 was effective in reducing synaptic depression and increasing synaptic facilitation. These results show that expression of CaBP1 and VILIP-2 can switch the direction of short-term plasticity from facilitation to depression and vice versa by altering $Ca_V2.1$-mediated Ca^{2+} currents without an effect on basal release probability (Sippy et al., 2003). The different effects of expression of VILIP-2 under conditions of low and high release probability highlight the intimate interplay between Ca^{2+} dynamics at the active zone and regulation of synaptic plasticity by CaS proteins in response to Ca^{2+} signals. Altogether, our results presented here show that the CaS proteins CaBP1 and VILIP-2 can serve as a bidirectional switch controlling synaptic facilitation and depression in a push-pull manner through opposing regulation of $Ca_V2.1$

channels.

CaS Proteins and Short-term Synaptic Plasticity

It is becoming ever more apparent that CaS proteins play a significant role in coupling changes in intracellular Ca^{2+} to modulation of different types of ion channels and other regulatory proteins. Ca^{2+} and CaM have long been implicated in postsynaptic mechanisms of synaptic plasticity (Chin and Means, 2000; Lisman et al., 2002; Pang et al. 2010). In addition, the CaS proteins frequenin, NCS-1, and K_V channel-interacting protein (KChIP), modulate a broad range of voltage-gated and ligand-gated channels (An et al., 2000; Nakamura et al., 2001; Tsujimoto et al., 2002; Zhang et al., 1998). Frequenin, a *Drosophila* NCS-1 homologue, has been shown to modulate the neurotransmitter release at the neuromuscular junction resulting in synaptic facilitation (Pongs et al., 1993). NCS-1, which is closely related to VILIP-2, enhances P/Q-type Ca^{2+} currents in the calyx of Held and can facilitate synaptic transmission at the calyx of Held and in hippocampal synapses similarly (Sippy et al., 2003; Tsujimoto et al., 2002), but modulation of $Ca_V2.1$ channels by direct binding of NCS-1 has not been observed. Our results indicate that CaBP1 and VILIP-2 can directly regulate short-term synaptic plasticity in SCG neurons in opposing ways by binding to the same regulatory site as CaM and differentially regulating $Ca_V2.1$ channels. The fact that these effects of CaS proteins on synaptic transmission are largely eliminated when mutant $Ca_V2.1_{IM\text{-}AA/\triangle CBD}$ channels mediate Ca^{2+} entry demonstrates the role of regulation of $Ca_V2.1$ channels by direct binding of CaS proteins as opposed to other possible presynaptic targets. Thus, regulated expression of these CaS proteins in different classes of synapses could work as a binary switch to change short-term synaptic plasticity from depressing to

facilitating or vice versa through direct modulation of $Ca_V2.1$ channels.

Distinct Expression of CaS Proteins in the Central Nervous System

CaS proteins are expressed in a cell-specific manner in the central nervous system (Burgoyne, 2007; Burgoyne and Weiss, 2001; Haeseleer et al., 2002; Paterlini et al., 2000). VILIP-2 is mainly expressed in the caudate-putamen, neocortex, and hippocampus (Braunewell and Gundelfinger, 1999; Paterlini et al., 2000). Expression of VILIP-2 is notably absent in the cerebellum and pons (Kajimoto et al., 1993; Paterlini et al., 2000). CaBP1 is primarily expressed in cerebral cortex, retina, and hippocampus in contrast to the ubiquitously expressed CaM (Haeseleer et al., 2002). Additionally, CaBP1 and $Ca_V2.1$ channels coexist in the CA1 region of the hippocampus and in presynaptic nerve terminals in the molecular layer of the cerebellum (Lee et al., 2002). Neuronal CaS proteins have Ca^{2+}-binding affinities considerably higher than CaM (Mikhaylova et al., 2011). Therefore, the diversity of this family of CaS proteins and their Ca^{2+} binding properties raises the possibility that they may contribute broadly to the diversity of Ca^{2+} channel regulation and synaptic plasticity.

CaS Proteins as Effectors of the Diversity of Synaptic Transmission

Different synapses show diverse patterns of facilitation and depression, and the underlying mechanism for this diversity is unknown (Zucker and Regehr, 2002). CaS proteins contribute substantially to the diversity of neuronal Ca^{2+} signaling in the presynaptic cell (Burgoyne, 2007). Our results provide a new perspective on the role of CaS proteins in synaptic plasticity by showing that CaBP1 and VILIP-2 can exert opposing influences on

facilitation and depression of synaptic transmission through regulation of presynaptic $Ca_V2.1$ channels. This new perspective opens the way for a broader analysis of the potential roles of other CaS proteins in control of short-term synaptic plasticity by regulation of presynaptic Ca^{2+} channels. Given the widespread distribution of $Ca_V2.1$ channels in the central nervous system, cell-specific modulation of presynaptic $Ca_V2.1$ channels by CaBP1, VILIP-2, and other CaS proteins may act as a molecular switch linking CaS-dependent facilitation and inactivation of $Ca_V2.1$ channels to encoding of information in the frequency domain at synapses.

Appendix A

A Cholinergic Model Synapse: dissection and microinjection of sympathetic superior cervical ganglion (SCG) neurons in long-term Culture

Summary

The cholinergic synapse formed between sympathetic superior cervical ganglion (SCG) neurons in long-term culture is an example of a fast synaptic model to explore the relationship between biochemical properties of a specific protein and its physiological significance in synaptic transmission. To explore the function in presynaptic terminals, only a few unique synapses such as the squid giant synapse, the calyx of Held synapse, and the hippocampal neuron autapse have been used (Eggermann et al. 2011). SCG synaptic cultures are special in that they are amenable to microinjection of cDNA in single presynaptic SCG neurons, which assures that only the presynaptic neuron is transfected. It is also possible to microinject large cDNAs encoding channel proteins that are otherwise difficult to express in central neurons. Thus, SCG synapses are ideal system to demonstrate the function role of presynaptic proteins in neurotransmitter release. Here, we describe the steps required to dissect neonatal sympathetic neurons from postnatal day seven Wistar rats for long-term culture. The following dissection protocol is modified from Mochida et al., 2003. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Washington.

Protocol

I. Preparation for dissection

1. Dissection media: prepare 50 mL L-15 Medium (Leibovitz, Invitrogen) with 5% Pen-Strep (Penicillin Strepomycin, Sigma-Aldrich). Store at 4°C.
2. Culture media: prepare 500 mL, 84% Minimal Essential Medium (MEM, Invitrogen), 10% Fetal Bovine Serum (FBS, Invitrogen), 5% Horse Serum (HS, heat inactivated, Invitrogen), and 1% Pen-Strep. Store at 4°C.
3. Final media: Prepare 50 mL Culture Media supplemented with 25 ng/mL NGF final concentration (Neural growth factor, N-100, Alomone labs). Store at 4°C.
4. Equipment & Tools: Dissecting microscope with light source, two micro-dissecting stainless steel forceps (Dumont #5, Fine Science Tools), large scissors, fine spring scissors (Fine Science Tools), four dissection pins, dissection board (styrofoam block topped with paper towel), disposable transfer pipette, ice bucket. Access to a tissue culture incubator and a laminar flow culture hood.
5. Poly-D-lysine coated 25 mm coverslips: Prepare Poly-d-lysine (PDL) coated coverslips by diluting stock solution to 0.1 mg/mL in 10 mL dH_2O (from 1 mg/mL stock made up in dH_2O stored at 4 °C). In 100 mm culture dish, add PDL and 20-30 coverslips (25mm, sterile, Fisher 12-545-86), cover and place on shaker (medium speed) in 4 °C overnight. After 24 hours, aspirate PDL and rinse coverslips 2X with sterile water. In sterile hood, dry coverslips by placing them on a paper towel to absorb moisture. Once dry, coated coverslips may be stored in 100 mm culture dish layered with kim wipe for two weeks.

6. Enzyme solution: prepare enzyme solution just prior to the incubation step. Final concentration of enzyme dissolve in L-15 is 2.5 mg/mL collagenase Type I (Worthington). The exact concentration of collagenase is critically and varies between lots of enzyme. Thus, it must be determined empirically for each new lot. Collagenase powder should be stored at 4°C and made fresh the day of dissection.

II. Methods for Dissection

1. On postnatal day seven, neonatal Wistar pups are anesthesized with isofluorane and decapitated with sharp scissors, one pup at a time.
2. The head is placed ventral side up on a dissection board with end oriented toward you. Use sterile dissections pins to secure: push one pin through the roof of the mouth into the pad and push the second pin through trachea. Make midline longitudinal incision in the ventral neck skin with scissors. The skin is then retracted laterally and pinned to the bottom of the dissection board, thereby exposing the neck musculature.
3. Under a dissecting microscope, using two forceps (one in each hand) clear away skin and fat around the trachea, removing salivary glands and superficial neck muscles. The carotid artery is identified bilaterally and followed cranially until the carotid bifurcation is encountered (“Y” bifurcation; Figure 5.1). The superior cervical ganglion (SCG) lies on the medial side of the carotid bifurcation. Carefully dissect carotid bifurcation with SCG attached by severing the artery at the most cranial point, and carefully place in 60 mm culture dish containing cold dissection media. The SCG is an almond-shaped small mass of tissue loosely attached to the artery. Remove bilateral SCG. Collect SCG from full litter of pups ~10 pups = 20 ganglia.

4. Cleaning the Ganglia: Once ganglia have been isolated, they should be freed as much as possible of extraneous tissue. Under dissection microscope, remove pieces of carotid artery, fat, or other tissue. Work with forceps in each hand to tease away extra tissue leaving SCG intact.
5. Gently, working from the middle of the ganglia, lightly pinch surface to remove sheath. Pull away sheath from each end like removing a sock. Place cleaned ganglia into new small culture dish (60 mm) containing dissection media.
6. Once all SCG have been cleaned and desheathed. Cut away connective ends using small iris scissors, and hemi-cut SCG with 5-6 snips along edge of the ganglia perpendicular to the long axis of the ganglia. Hemi-cut is a snip that does not reach the edge of the ganglia leaving it intact.
7. Transfer ganglia with transfer pipet into 15 mL sterile conical tube. At this point, the ganglia are ready for enzyme dissociation.

III. Enzymatic Dissociation and Culture:

1. Remove excess L15 from conical tube. Add 1 mL 2.5 mg/mL collagenase Type I dissolved in L15 media to wash. Let tube rest. Remove supernatant and add another 1 mL 2.5 mg/mL collagenase Type I in L15 media. Incubate at 37 °C, 5% CO_2 humidified incubator for 25 min. Triturate 10Xs with fire-polished Pasteur pipet (large bore) then return to incubator for 25 min. Triturate 20Xs with fire-polished Pasteur pipet (small bore). Continue to triturate until no large clumps are visible and you reach a cloudy suspension. Add 10 mL complete L15 media to dilute out and neutralize the enzymes and centrifuge at 1300 rpm for 3 min. Discard supernatant,

rinse with Culture Media and centrifuge at 1300 rpm for 3 min. Discard supernatant and resuspend in 2 mL of Final Media.

2. Filter cells through 70 μm nylon cell strainer (BD Biosciences) into a new tube to remove any remaining clumps. If there is little debris, you may let the clumps settle and remove the supernatant into a new tube leaving behind about 100 μL at the bottom of the tube.
3. Add Final Media to bring total volume to 3 mL (The final volume depends on the density and number of dishes to be made). Aliquot 100-200 μL of cells on center of prepared coverslip in 35 mm culture dish and let settle for 1 hour. Total number of dishes depends on yield of cells, approximately 1 ganglion per coverslip.
4. After 1 hour, add 2 mL Final Media (MEM+NGF). Place in incubator for 4-6 weeks set at 37 °C, 5% CO_2 humidified incubator.
5. Maintenance of culture: replace 1mL MEM+NGF twice a week, using a fresh pipette tip for every dish to avoid contamination. After 4 weeks, cells are ready to measure synaptic transmission.

IV. Notes

1. The enzyme concentration must be adjusted for each lot of collagenase. Thus, enzymes should be purchased in large quantities. If large clumps of tissue remain after digestion, a higher concentration of enzyme is required. Large clumps will overgrow and make it difficult to access cells.

V. Preparation for Microinjection

This section describes the preparation steps that need to be taken prior to injecting cDNA into the nucleus of the neuron.

1. DNA encoding the protein of interest is subcloned into a suitable mammalian expression vector, pcDNA3, under the regulation of the constitutively active cytomegalovirus (CMV) promoter. If the protein is not labeled, a reporter plasmid encoding enhanced green fluorescent protein (EGFP) is coinjected to confirm successful injection and expression.
2. Immediately prior to microinjection, 5 μL of 5% Fast Green Dye (FGD; stock diluted in dH_2O) and plasmid cDNAs are diluted and mixed to the desired final concentration in dH_2O. Spin down the cDNA by centrifuge.
3. Typically, 5-10 ng/μl of cDNA is sufficient to label injected cells. It is recommended that the total concentration of cDNA be below 100 ng/μl since DNA is viscous at high concentrations and can clog injection pipets.
4. Glass pipets: Filamented, thin-walled borosilicate glass capillary tubes 1.0 outer diameter, length depends on electrode holder (World Precision Instruments, Sarasota, FL).
5. Pulling pipets: We use a one stage program to pull narrow microinjection pipet tips. Test the opening using 5% fast green dye (FGD) directly with pressure injection. Pull several injection pipets and store in a covered container to prevent dust from entering the microinjection pipet tip.
6. Intranuclear injection equipment: One may use different types of pressure injectors, for example Eppendorf FemtoJet Express (Lu et al., 2009). We use a hand-held

syringe pressure to inject SCG neurons. A 200 μl pipet tip is attached to a 1 ml syringe via parafilm to ensure no leaks in airflow. The fabricated syringe with tip is then inserted into tubing attached to the open valve of the electrode holder.

7. Loading the micropipet: A fresh, sterile, 1 ml syringe is flamed at the tip and pulled to make a filling syringe. Draw up cDNA no more than 5 mm from tip of filling syringe. Make sure to pull cDNA from the surface of the tube. Fill microinjection pipet. The filament will ensure that the cDNA solution reaches the tip. Tap the pipet to remove any visible bubbles.
8. Injection Procedure: Once microinjection pipet is ready, select target cell, and place electrode above the cell. At four weeks in culture, the nucleus of SCG is easily visible with one to two nucleoli (Figure 5.2*A*). For the injection step, slowly add positive pressure to ensure flow of cDNA solution and visibility of FGD. Inject the nucleus by pressing on the hand-held syringe and slowly entering the cell. The nucleus will turn green if the tip has penetrated the nuclear membrane. If the cytoplasm turns green or cell body swells, this is indicative of a missed nuclear injection. A second, deeper injection is needed to access the nucleus. A successful injected nucleus will appear dark green in color. The same pipet may be used if no clogging occurs. Continue to inject neurons in the same manner repositioning above the nucleus of the cell. Systematically navigate through the dish to avoid injecting the same cell as dye may fade. Successfully injected neurons can be identified in 24 hours post-injection by expression of the reporter gene, EGFP.

VI: Notes

1. Successful nuclear injections require the cells adhere to tissue culture plates. SCG neurons may be injected the same day as initial plating but one must allow for a few hours for SCG neurons to attach. For cell body current recordings using whole-cell voltage clamp, it is recommended to inject and record within a week of plating. SCG neurons grow processes and become less spherical with time in culture making it difficult to control the voltage-clamp during recordings.
2. Clogging of pipets may be a problem. Between injections it is recommended to spin down tubes containing cDNA. If clogging persists, a new pipet should be used. If clogging persists, consider changing the parameters of the micropipette puller to make a microinjection pipet tip with a large opening, since micropipette pullers may be unstable with a single pull program.
3. When starting to practice intranuclear microinjections it is best to start with the reporter gene before doing actual experiments.

Conclusions

SCG neurons in long-term culture are advantageous in the study and manipulation of presynaptic proteins. However, successful cultures and intranuclear microinjections are technically demanding and require patience. Proficiency with this preparation comes with practice and a high degree of troubleshooting during both the culturing procedure and microinjection. Furthermore, both preparation of SCG neuronal synaptic cultures and intranuclear injections have a low success rate. Despite its drawbacks, the technique is useful for heterologous expression of proteins in neurons and the ability to study the effect of a

protein in a functional system is extremely significant. The ability to functionally express large proteins, like $Ca_V2.1$ channels, and have the neuron express these channels at presynaptic terminals is profound.

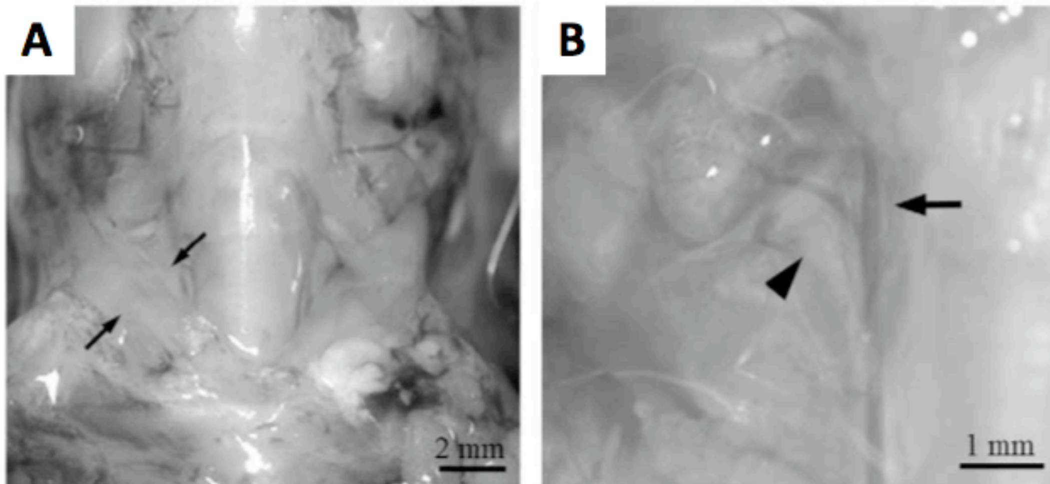


Figure 5.1. Dissection of superior cervical ganglion from neonatal rat.

The two large salivary glands along the midline were exposed after the neck skin was retracted. **(A)** After the salivary glands were removed, the neck muscles (bracketed by arrows) are revealed on both sides. **(B)** Transection of the muscles exposes the carotid artery and its bifurcation. The nearby nodose ganglion (indicated by the arrowhead) is located directly lateral to the branch point of the carotid artery, and the sympathetic ganglion (indicated by the arrow) is located medially beneath the carotid bifurcation. Adapted from He and Baas, 2003.

A

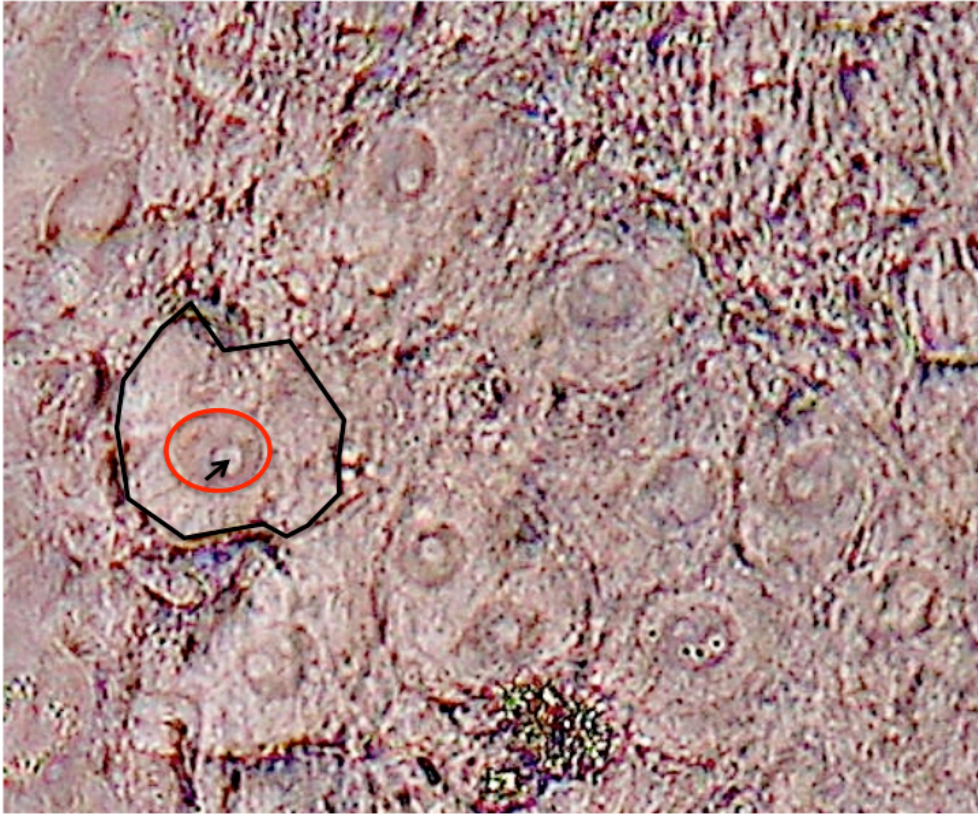


Figure 5.2. Sympathetic superior ganglion neurons in culture.

(A) Representative image of SCG neurons in 4 weeks culture. A single SCG neuron is outlined in black. The large nucleus of SCG neurons appears darker against the cytoplasmic region (outlined in red). The arrowhead denotes the presence one nucleolus in the nucleus. SCG neurons grow in clusters from 6 to 10 per cluster with supporting mitotic cells.

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Zhou, H., Kim, S.A., Kirk, E.A., Tippens, A.L., Sun, H., Haeseleer, F., and Lee, A. (2004). Ca^{2+}-binding protein-1 facilitates and forms a postsynaptic complex with $Ca_V1.2$ (L-type) Ca^{2+} channels. J Neurosci *24*, 4698-4708.

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Zucker, R.S., and Regehr, W.G. (2002). Short-term synaptic plasticity. Annu Rev Physiol *64*, 355-405.

Curriculum Vitae

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EDUCATION

University of Washington, Seattle, WA
Doctorate of Philosophy, Neurobiology and Behavior, 2012
Advanced to candidacy December 2008
Thesis: Fine-tuning Synaptic Plasticity by Modulation of Presynaptic Ca_V2.1 Channels with Ca^{2+} Sensor Proteins

Brown University, Providence, RI
Bachelor of Science, Neuroscience, 2000 - 2004

HONORS/AWARDS

University of Washington
Institutional Training Grant for Neurobiology, (NIH-T32 GM07108).
September 2007- June 2010

University of California, Los Angeles
Research Supplement for Underrepresented Minorities (NINDS-NS4764-02S1).
December 2004 - August 2005 & December 2005 - August 2006

RESEARCH EXPERIENCE & TRAINING

University of Washington
Ph.D. Neurobiology and Behavior Program
September 2006 – June 2012

Laboratory of Dr. William A. Catterall

Research objectives:

- To establish a new model synapse model using sympathetic superior cervical ganglion (SCG) neurons. SCG neurons in long-term culture form fast cholinergic synapses amenable to microinjection manipulations for the study of presynaptic proteins.
- To elucidate the potential connections between neuronal calcium sensors and regulation of $Ca_v2.1$ channels in short-term synaptic plasticity primarily using electrophysiology (whole-cell voltage patch clamp and sharp intracellular recordings).
- To delineate mechanisms of neurotransmitter release through facilitation and/or inactivation of $Ca_V2.1$ channel currents by CaBP1 and VILIP-2 and determine their role in synaptic transmission.

University of California, Los Angeles
Research Assistant, Department of Physiological Science
July 2004 - August 2006
Laboratory of Dr. Marc Klein

Research objectives:

- To characterize the role of calcium-dependent protein kinase C in synaptic plasticity in *Aplysia Californica*. Primarily using electrophysiology and fluorescence imaging, this research examined the translocation of protein kinase C in response to action potential firing in the presynaptic sensory neuron *Aplysia* in sensory-motor synapses.

TEACHING

University of Washington
Graduate Teaching Assistant, Neurobiology and Behavior Program
January 2009 – March 2009

- Instructor for undergraduate Introduction to Neurobiology 301 laboratory course
- Taught weekly electrophysiology laboratory sections for undergraduate introductory neurobiology course, which included dissections, intracellular, and extracellular recordings.
- Advised undergraduate students during office hours, graded exams, and assignments

PUBLICATIONS

Leal, K., Mochida, S., Scheuer, T., Catterall, W.A. (2012). Fine-tuning synaptic plasticity by modulation of presynaptic $Ca_V2.1$ channels with Ca^{2+} sensor proteins. ***Submitted, Neuron*.**

Suh, B.C., **Leal, K**., Hille, B. (2010). Modulation of high-voltage activated Ca^{2+} channels by membrane phosphatidylinositol 4,5-bisphosphate. ***Neuro**n*. 67:224-238.

Leal, K and Klein, M. (2009). Direct enhancement of presynaptic calcium influx in presynaptic facilitation at *Aplysia* sensorimotor synapses. ***Mol Cell Neurosci.*** 41(2): 247-57.

Zhao, Y., **Leal, K.**, Abi-Farah, C., Martin, K.C., Sossin, W.S., and Klein, M. (2006). Isoform Specificity of PKC Translocation in Living *Aplysia* Sensory Neurons and a Role for Ca^{2+}-Dependent PKC APL I in the Induction of Intermediate-Term Facilitation. ***J Neurosci.*** 26: 8847-8856.

ABSTRACTS AND PRESENTATIONS

Catterall, W.A., Sudhof, T.C., **Leal, K.**, Lipscombe, D. (2012). Calcium channel regulation and presynaptic plasticity. 45th Annual Winter Conference on Brain Research. Snowbird, UT

Leal, K., Mochida, S., Scheuer, T., Catterall, W.A. (2010). Presynaptic calcium sensor protein dependent modulation of $Ca_v2.1$ channels and synaptic plasticity. Society for Neuroscience, San Diego, CA.

Leal, K., Abi-Farah, C., Zhao, Y., Martin, K.C., Sossin, W.S., and Klein, M. (2005). Mobilization of calcium-dependent protein kinase C by concurrent activity and serotonin in *Aplysia* sensory neurons. Society for Neuroscience, Washington D.C.

SERVICE AND MEMBERSHIPS

Member, Society for Neuroscience, 2005 - Present
Member, Neurobiology and Behavior Outreach, University of Washington, 2006 - Present
Volunteer, Brain Awareness Week, University of Washington, 2006 – Present