NEUROMUSCULAR TRANSMISSION IN A NATURALLY OCCURRING MOUSE MUTANT OF THE β SUBUNIT OF THE NEURONAL CALCIUM CHANNEL

BY

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A DISSERTATION

Submitted to
Michigan State University
In partial fulfillment of the requirements
For the degree of

DOCTOR OF PHILOSOPHY

GENETICS

2011

ABSTRACT

NEUROMUSCULAR TRANSMISSION IN A NATURALLY OCCURRING MOUSE MUTANT OF THE β SUBUNIT OF THE NEURONAL CALCIUM CHANNEL

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Entry of Ca^{2+} through voltage gated calcium channels (VGCCs) into nerve terminals is a necessary step coupling the action potential to release of acetylcholine (ACh). VGCCs are heteromultimeric complexes of α_1, $\alpha_2\delta$, and β subunits, and sometimes γ subunits. The specific α_1-β combination assembled determines the channel properties. The mouse mutant *lethargic* (*lh*) has severe neurological defects due to a mutation that deletes α_1 subunit interaction domain of the β_4 subunit. β_4 normally associates with the α_{1A} subunit of the P/Q-type VGCCs, and has a major role in stabilizing the final α_{1A} subunit conformation and targeting it to the cell membrane. Loss of the β_4 subunit could alter the channel characteristics and localization of α_{1A}. The overall goal of this dissertation was to test the hypothesis that disruption of the β_4 subunit affects the function of the α_{1A} subunit of the P/Q-type VGCCs.

Electrophysiological recordings were performed at neuromuscular junctions (NMJs) of adult *lh* and *wild type* (*wt*) mice. The quantal content and phrenic nerve evoked release showed a significant decrease in *lh* with respect to *wt*. The frequency of

spontaneous release of ACh also decreased significantly, although the reduction was only evident when Ca^{2+} was replaced by Sr^{2+} or Ba^{2+} as charge carriers. The amplitude of spontaneous release was not affected by this mutation, implying that each vesicle contains approximately the same amount of ACh in *wt* and *lh* mice. These results are due to a significantly slower process of neurotransmitter vesicles release, as confirmed by FM1-43 staining method.

There are specific VGCCs antagonists that can be used to determine the contribution of the different types of VGCCs in nerve-stimulated ACh release from motor nerve terminals. ω-agatoxin IVA and SNX-482, specific antagonists for P/Q- and R-type VGCCs respectively, significantly reduced the quantal content in adult *lh* mice. Immunolabeling of VGCC subunits revealed an increase in α_{1E}, β_1 and β_3, but no apparent change in the levels of α_{1A} at adult *lh* neuromuscular junctions. Therefore, *lh* animals control ACh release by P/Q- and R-type VGCCs.

The studies of this dissertation provide evidence for: 1) decreased nerve-evoked ACh release in *lh* mice, 2) slowed vesicle release process in *lh* mice, 3) increased level of β_1 and β_3, compensating for the lack of β_4 subunit, and 4) P/Q- and R-type VGCC involvement in release of ACh from motor nerve terminals.

To Gastón, a great brother, who always had faith in me.

ACKNOWLEDGEMENTS

First and foremost I would like to thank my advisor, Dr. William Atchison for his invaluable guidance during my graduate career. His support, advice and guidance have helped me become an independent researcher. I could not have chosen a better mentor to provide the right amount of oversight and independence necessary for my success as a student and researcher. I would also like to thank my committee members, Dr. Ke Dong, Dr. John Fyfe, and Dr. Colleen Hegg. Their expertise, ideas, technical assistance and thoughtful critiques were instrumental in the development and progression of this research project.

I also want to acknowledge Ms. Jeannine Lee and Dr. Barbara Sears, from the genetics program, for all their help, guidance and assistance. Dr. Stephen Schneider helped in correcting and editing my dissertation, and Dr. Nicole Pardo a previous member of Dr Atchison's lab, who guided me through electrophysiology and her assistance in various aspects of my research, Dr. Ravindra Hajela for his help in genotyping animals, and Dr. Yukun Yuan for all his valuable advice regarding my research, as well as Ms. Dawn Autio for her incredible support and guidance with immunohistochemistry. I must acknowledge my lab mates, Brenda Marrero-Rosado, Sara Fox, Aaron Bradford, Alexandra Colon, Heidi Hannon, Dr. Wen-hsin Ku, Dr. Frank Johnson, Dr. Erin Wakeling, and Dr. Sameera Dasari, and the girls from Puerto Rico (Desiree, Maryvi, Angie, Chesky, Katiria and Rosa) for making the lab a nice enjoyable place and keeping me sane. To all my friends here and elsewhere, sincere thanks for your

love and support. I am eternally thankful to Gabriel, for his love, incredible patience, help and for always being by my side in the hardest times; graduate school would not have been the same without you. Finally, I am forever thankful to my family, Mom, Dad, Florencio, Martina and Gastón for always being there by my side, thank you for all your love and support despite being so far away from home. I could not have done this without you.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ABP	AID-binding pocket
ACh	Acetylcholine
AChE	Acetylcholinesterase
Aga-IVA	ω -agatoxin IVA
AID	α-interaction domain
α-LTx	α-latrotoxin
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
α-SNAP	α-soluble NSF attachment protein
BCA	Bicinchoninic acid
BID	β-interacting domain
ChAT	Choline-O-acetyl-transferase
CNS	Central nervous system
Ctx GVIA	ω-conotoxin GVIA
DAP	3,4-diaminopyridine
FITC	Fluorescein isothiocyanate
EDL	*Extensor digitorum longus*
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ER	Endoplasmic reticulum
EPP	End-plate potential
GABA	γ-aminobutyric acid

GK	Guanylate kinase
Glu	Glutamate
Gly	Glycine
HEPES	2-hydroxyethyl-1-piperazineethanesulfonic acid
HVA	High voltage activated
IHC	Immunohistochemistry
$[Ca^{2+}]_i$	Intracellular calcium concentration
LEMS	Lambert-Eaton myasthenic syndrome
lh	*Lethargic*
LTP	Long term potentiation
LVA	Low voltage activated
m	Quantal content
MEPP	Miniature end-plate potential
nACh	Nicotinic acetylcholine
NMDAR	N-methyl-D-aspartate receptor
NMJ	Neuromuscular junction
NSF	N-ethylmaleimide-sensitive factor
PBS	Phosphate-buffered saline
SDS PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SH3	Src homology 3
SNAP-25	Synaptosomal protein of 25 kD

SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptors
stg	*Stargazer*
Synprint	Synaptic protein interaction
TARPs	Transmembrane AMPA receptor regulatory proteins
tg	*Tottering*
tg^{4J}	*Tottering 4 Jackson*
tg^{5J}	*Tottering 5 Jackson*
tg^{la}	*Tottering leaner*
tg^{rol}	*Tottering rolling Nagoya*
TS	*Triangularis sterni*
VGCCs	Voltage gated calcium channels
wt	W*ild type*

CHAPTER 1

INTRODUCTION

A. General Introduction

Voltage gated calcium channels (VGCCs) contribute to the entry of Ca^{2+} into nerve terminals. This is a necessary step coupling the action potential to release of acetylcholine (ACh) (Augustine et al., 1987; Llinas et al., 1976; Katz and Miledi, 1970). Although multiple VGCCs subtypes are known to coexist in the same cell, the specific channel subtype involved in release of ACh from motor nerve terminals is both species- and age-dependent (Catterall, 1998; Rosato Siri and Uchitel, 1999). During early stages of development, motor nerve terminals have multiple subtypes of VGCCs (Rosato Siri and Uchitel, 1999; Santafe et al., 2001). Mature motor nerve terminals, however, contain primarily one subtype of VGCCs involved in the release of ACh. Mature mammalian motor nerve terminals utilize P/Q-type ($Ca_V2.1$) (Katz et al., 1995), whereas amphibians (Sano et al., 1987) and birds (De Luca et al., 1991) rely mainly on N-type ($Ca_V2.2$) VGCCs to control the release of ACh. However the complement of VGCCs does not necessarily seem to be fixed. Under specific conditions, subtypes of VGCCs that are not normally associated with ACh release at motor nerve terminals can mediate it (Flink and Atchison, 2002; Pardo et al., 2006).

VGCCs are categorized into two main classes depending on the extent of depolarization from rest needed to activate them. These are: low voltage-activated and high voltage-activated VGCCs. The former are activated by small depolarizations from rest, and hence open at highly negative membrane potentials, while the latter require strong depolarizations and activate at more depolarized membrane potentials.

The different VGCCs can be distinguished by the genes that encode them and their pharmacological and biophysical characteristics (Catterall et al., 2005; Zhang et al., 1993). VGCCs are formed by α_1, β, and $\alpha_2\delta$ subunits (Tsien et al., 1991). Some also contain a γ subunit. The α_1 subunits make up the selective pore for Ca^{2+} and determine most of the subtype-specific attributes of VGCCs. They contain binding sites for various pharmacological agents as well as the gating regions of the channel (Catterall, 1995; Zhang et al., 1993). There are at least five α_1 subunits for neuronal VGCCs which fall under the high voltage activated (HVA) subclass. They include: α_{1A}, α_{1B}, and α_{1E} subunits which represent the P/Q- ($Ca_V2.1$), N- ($Ca_V2.2$), and R-type ($Ca_V2.3$) VGCCs, respectively; while the α_{1C}, α_{1D}, α_{1F} and α_{1S} represent the L-type channels ($Ca_V1.2$-1.3) (Catterall, 1995; Tsien et al., 1991).

In VGCCs the β subunit is a cytoplasmic protein that regulates the assembly and membrane localization of the α_1 subunits (Dolphin, 2003). The β subunit also strongly influences the current amplitude, rate and voltage-dependence of activation and inactivation, and ligand-binding sites on the channel (Brice and Dolphin, 1999; Catterall, 1995; Dolphin, 2003; Walker and De Waard, 1998). There are four different types of β subunits ($\beta_{1\text{-}4}$) which each are encoded by different genes (Chien et al., 1995). The β_4 subunit is a common constituent of neuronal P/Q- VGCCs (Wittemann et al., 2000). The correct β subunit is essential for its interaction with its corresponding α_1 subunit for proper targeting, membrane insertion, channel density, kinetic parameters such as

activation and inactivation, as well as interactions with vesicular release site proteins (Murakami et al., 2003; Wittemann et al., 2000). However, in the absence of the normally associating β subunit, alternate β subunits may interact with α_1 subunits to restore most of the VGCCs' functions, although in an altered manner (Burgess et al., 1999).

The β_4 subunit, which typically associates with the α_{1A} subunit, is normally widely expressed in the brain. It has been reported that spontaneous mutations in this subunit cause several neurological syndromes in mice (Burgess et al., 1997; Catterall, 1995); it produces various effects in VGCC expression and function, such as dramatically reducing the VGCCs' targeting, assembly, membrane insertion, and channel density. It also alters the characteristic kinetic parameters, vesicular release and synaptic transmission (Burgess et al., 1999; Catterall, 1995; Catterall et al., 2005; Walker et al., 1998). Also, the loss of a functional β_4 subunit can impact the function of α_{1A} − containing VGCCs (P/Q-type) (Helton and Horne, 2002).

A four base pair insertion into a splice donor site within the β_4 gene in mouse chromosome 2 leads to the lethargic (*lh*) mutation. This insertion leads to exon skipping, translational frameshift, and protein truncation, with omission of 60% of the C-terminal of the β_4 subunit relative to wild type (*wt*), including loss of the α_1-binding site. This suggests that a defect in VGCC assembly could be one cause for the pathogenesis in *lh* phenotype (Burgess et al., 1997; Burgess et al., 1999). The *lh* mice suffer from ataxia, lethargic behavior, spike-wave epilepsy, and paroxysomal dyskinesia. The onset of ataxia is two weeks after birth (Khan and Jinnah, 2002). Electrophysiologically and pharmacologically, these seizures are similar to the *absence* seizures present in the

human *petit mal* epilepsy and to those present in tottering (*tg*) mice (Burgess et al., 1999; Hosford et al., 1992). In addition to the neurological signs, *lh* mice show reduced body weight and immunological problems when compared with unaffected litter mates (Sidman et al., 1965).

B. Neuromuscular Junction and Vesicle Release

The neuromuscular junction (NMJ) is a chemical synapse that occurs between the axons of lower motor neurons of the spinal cord or brainstem neurons and skeletal muscle. It is comprised of a specialized cleft or synapse (which is 20 – 30 nm wide) between motor nerve terminals and the muscle's end plate, where nicotinic ACh (nACh) receptors are clustered in junctional folds. Neuromuscular synaptic transmission is fast and reliable. An action potential in the motor axon, leads to the release of chemical neurotransmitters from the motor nerve terminals causing an action potential in the muscle cell leading to muscle contraction. This reliability is accounted for, in part, by structural specializations of the NMJ. The presynaptic terminal contains a large number of highly specialized regions known as active zones, which are the sites of neurotransmitter release in the presynaptic membrane (Ceccarelli et al., 1979; Dreyer et al., 1973; Heuser et al., 1974; Heuser et al., 1979; Heuser and Reese, 1981; Rash et al., 1974). In addition, the post-synaptic membrane at the motor end-plate contains a series of shallow folds packed with neurotransmitter receptors. The NMJ contains several proteins which are involved in mediating the connections between the motor nerve endings in the presynaptic region with the muscle's end plate in the postsynaptic region.

Fast synaptic transmission at most central nervous system (CNS) synapses is mediated by glutamate (Glu), γ-aminobutyric acid (GABA), and glycine (Gly). The motor nerve terminal contains the chemical neurotransmitter ACh, which also mediates fast synaptic transmission. Chemical synaptic transmission requires that neurotransmitters be synthesized and ready for release. Neurons contain specific enzymes that synthesize neurotransmitters from various metabolic precursors. The synthesizing enzymes for both amino acid and amine neurotransmitters are transported to the axon terminal, where they locally and rapidly direct transmitter synthesis. Once synthesized in the cytoplasm of the axon terminal, amino acid and amine neurotransmitters must be taken up by synaptic vesicles through transporters. ACh is synthesized in the terminals from acetyl-CoA and choline. This reaction is catalyzed by choline-O-acetyl-transferase (ChAT) (Browning and Schulman, 1968; Hebb, 1972; Tucek, 1982). Once ACh is synthesized it is transported to synaptic vesicles for storage and release via an ATPase-dependent transport system (Anderson et al., 1982; Breer et al., 1977; Parsons and Koenigsberger, 1980). There are two populations of synaptic vesicles. Those vesicles that are immediately available for synaptic transmission, the "readily releasable pool", are found close to the nerve terminals in the active zone (the regions involved with neurotransmitter release). The other population includes those vesicles that serve to replenish the readily releasable pool once it is empty and are known as the "reserve pool".

This movement of vesicles between both populations of vesicles is known as "mobilization" (Delgado et al., 2000; Kuromi and Kidokoro, 2002; Richards et al., 2000). It is believed that synapsin, which is a synaptic vesicle protein, is involved in this process

by a series of phosphorylation / dephosphorylation – dependent interactions with cytoskeletal proteins such as F-actin (Humeau et al., 2001; Llinas et al., 1991; Petrucci and Morrow, 1987). In the case of cholinergic synapses, the refilling of the readily releasable pool occurs by synthesis of new ACh (Collier, 1986). However, it is not an all-or-nothing process, there is a process known as "kiss and run" in which there is incomplete release of vesicles. This process has been measured at the NMJs, hippocampal synapses and chromaffin cells. (Ceccarelli et al., 1973; Fesce and Meldolesi, 1999; Klingauf et al., 1998; Kraszewski et al., 1996; Palfrey and Artalejo, 1998; Verstreken et al., 2008; Zefirov et al., 2004).

ACh is released in defined packets known as quanta (Boyd and Martin, 1956; del Castillo and Katz, 1954; Katz and Miledi, 1967b), in which each synaptic vesicle is believed to represent a single quantum of ACh (Heuser and Reese, 1973; Heuser et al., 1979). The ACh release can either be spontaneous or induced by an action potential. The spontaneous release of ACh represents single packets of quanta that are released asynchronously. These single packets produce small depolarizations at the end-plate region of the muscle, known as miniature end-plate potentials (MEPP) (Katz and Miledi, 1963, 1967b). MEPPs have an amplitude distribution around mean amplitude equal to that of a small end-plate potential (EPP). These small depolarizations are not enough to reach threshold, and so an action potential in the postsynaptic membrane does not occur (Sellin et al., 1996). Action potentials evoke ACh release of several quanta from the motor nerve terminal in a synchronous way; this results in a large depolarization of the muscle membrane, known as an EPP (Boyd and Martin, 1956; del Castillo and Katz, 1954; Fatt and Katz, 1951).

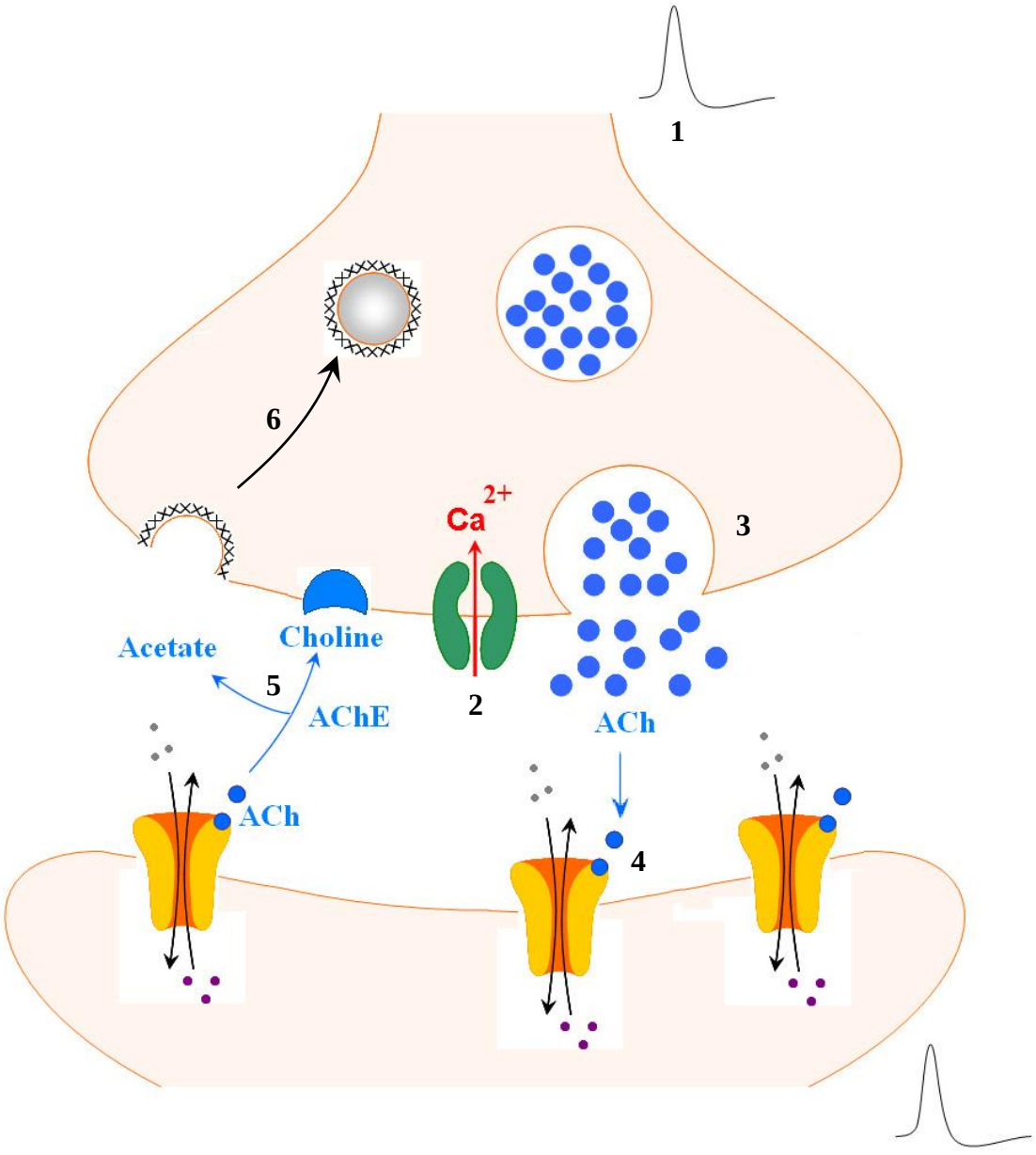
An action potential in the nerve terminal, produced by the movement of Na^+ and K^+ down their respective electrochemical gradients, invades the terminal membrane (Fig. 1.1). This action potential eventually ceases near the end of the axon, but the currents spread electrotonically and depolarize the membrane (Mallart and Brigant, 1982; Mallart, 1985a). As a result of membrane depolarization, VGCCs open, allowing Ca^{2+} to move down its electrochemical gradient from the extracellular environment into the nerve terminal (Augustine et al., 1987; Katz and Miledi, 1967a, 1970; Llinas et al., 1981a). This rapid increase in intracellular [Ca^{2+}] occurs in distinct domains at active zones (Llinas et al., 1992) and induces synaptic vesicle fusion with the surface membrane, releasing the neurotransmitter molecules into the synaptic cleft (Ceccarelli et al., 1979; Ellisman et al., 1976; Harris and Sultan, 1995; Heuser et al., 1974; Heuser et al., 1979).

Even though neurotransmitter release is triggered by Ca^{2+} entry via VGCCs in nerve endings, it is not the only factor required for synaptic vesicle exocytosis. The delay observed between presynaptic Ca^{2+} influx and the changes produced by the ACh in the end-plate is very short, as little as 200 μs (Llinas et al., 1981b; Sabatini and Regehr, 1996). The rapidity of this response led to the supposition that fusion of synaptic vesicles with the plasma membrane was already preformed prior to Ca^{2+} entry into the nerve terminal (Jahn and Südhof 1999; Lonart and Südhof, 2000). Moreover, there can be release of ACh in the absence of Ca^{2+}. The release can be induced by high osmolarity conditions or in the presence of α-latrotoxin (α-LTx) (Hubbard et al., 1968; Rosenmund

Figure 1.1: Neuromuscular transmission.

(1) Stimulation of the nerve leads to initiation and propagation of an action potential. (2) Depolarization of presynaptic terminal leads to opening of VGCCs, which allow rapid influx of Ca^{2+} to the nerve terminal. (3) Ca^{2+} causes synaptic vesicles to fuse with the synaptic membrane. The neurotransmitter (ACh) is released to the synaptic cleft via exocytosis. (4) Two ACh molecules bind to receptor molecules in the postsynaptic membrane (nicotinic ACh receptors), leading to the opening of the ACh receptor associated channel located on the end-plate region of the muscle. Diffusion of Na^{+} and K^{+} across the channel causes depolarization which opens voltage gated sodium channels in the adjacent muscle membrane which allows for muscle action potential and muscular contraction. (5) The effects of ACh are rapidly terminated by the action of AChE, which hydrolyzes ACh into acetate and choline. Choline is taken back into the cytoplasm of the nerve terminal by a high affinity transporter. Choline is then combined with acetylCoA by the enzyme choline-O-acetyltransferase (ChAT) to form new molecules of ACh, which are then stored in the synaptic vesicles via an ATP-dependent transport system. (6) Recycling of vesicular membrane from plasma membrane occurs through a clathrin-mediated cycle. (Adapted from Purves et al., 2008)

Figure 1.1 (cont'd)



For interpretation of the reference to color of this and all other figures, the reader is referred to the electronic version of this dissertation.

and Stevens, 1996). α-LTx is a component of the black widow spider venom, and produces massive exocytosis of synaptic vesicles. Additionally Ba^{2+} and Sr^{2+} readily enter the cell through VGCCs and can support the generation of MEPPs in the absence of Ca^{2+} (Silinsky, 1977; 1978; 1981; 1985). However, Sr^{2+}, but not Ba^{2+} can also replace Ca^{2+} to support an EPP in the process of synchronous-evoked release. Using Ba^{2+} and Sr^{2+} for neurotransmitter release at the squid synapse has shown that the release is much lower than for an equivalent amount of Ca^{2+} influx. The amplitude of post-synaptic current elicited by presynaptic transmitter release and the amplitude of postsynaptic potential changes was reduced when Ca^{2+} was replaced with equivalent concentrations of Sr^{2+} or Ba^{2+} (Augustine and Eckert, 1984). This suggests that the ion-sensitive step in the release process prefers Ca^{2+} strongly over other ions in the order of $Ca^{2+} > Sr^{2+} > Ba^{2+}$ (Dodge et al., 1969; Meiri and Rahamimoff, 1971; Miledi, 1966). However, Ba^{2+} and Sr^{2+} cause a much higher MEPP frequency than Ca^{2+} (Mellow et al., 1982; Silinsky, 1978; 1981). Therefore, it seems that Ca^{2+} most likely acts as a key regulator, but not an absolute requirement for exocytosis.

There are four molecules that are central to the exocytosis of synaptic vesicles. They are synaptotagmin, synaptobrevin (this is the only synaptic vesicle protein), syntaxin and SNAP-25 (synaptosomal associated protein of 25 kDa). Fusion of synaptic vesicles with the plasma membrane involves the formation of stable core complexes of proteins known as a SNARE [Soluble N-ethylmaleimide-sensitive factor (NSF)

Attachment Protein Receptors] complex. SNAREs consist of two groups. The nerve terminal or t-SNAREs are composed by SNAP-25 and syntaxin, and are localized on the plasma membrane. The synaptic vesicle or v-SNARE, is composed of synaptobrevin, and found on vesicle membranes (Bennett et al., 1992a; Bennett et al., 1992b; Oyler et al., 1989; Trimble and Scheller, 1988). SNAREs have been shown to mediate membrane trafficking and secretion in both mammalian and yeast cells (Fig 1.2) (Bennett et al., 1992a; Bennett et al., 1992b). The formation of the core complex is a highly regulated event in which a protein known as munc18 is involved (Hata et al., 1993; Pevsner et al., 1994). This is a highly soluble protein found in the nerve endings. It is not part of the fusion complex, however it is able to bind syntaxin, and this binding reaction prevents syntaxin from joining the SNARE complex (Misura et al., 2000; Pevsner et al., 1994; Weber et al., 1998). Once munc18 dissociates, a highly stable core complex is formed in such a manner that promotes a condition amenable to synaptic vesicle and plasma membrane fusion. The interaction between synaptobrevin, syntaxin and SNAP-25 is a very tight one. For transmitter release to proceed normally, the SNARE complex must become disassembled. This process is carried out by the binding of NSF along with the α-soluble NSF accessory protein (α-SNAP) (Sollner et al., 1993).

The SNARE complex (v- and t- SNARE) mediates the fusion of synaptic vesicles to the plasma membrane resulting in exocytosis. Syntaxin (member of membrane integrated Q-SNARE protein complex participating in exocytosis) and synaptotagmin (Ca^{2+} sensor synaptic vesicle protein) have been shown to co-localize with N-type VGCCs (Martin-Moutot et al., 1993) indicating that VGCCs interact with proteins involved in the release apparatus. Direct interaction of N- and P/Q-type VGCCs is known

Figure 1.2: Configuration of the interactions of synaptic vesicle and plasma membrane proteins before (a) and after (b) the formation of the SNARE complex. (Adapted from Levitan and Kaczmarek, 1997)

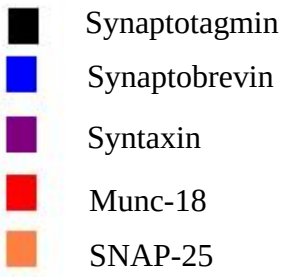
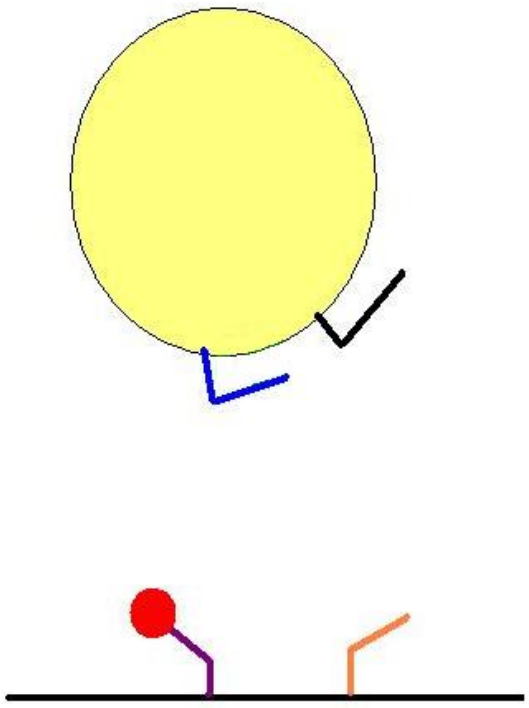
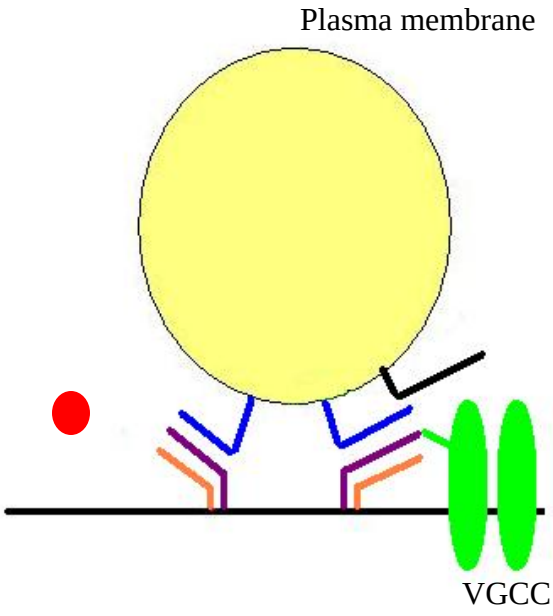


Figure 1.2 (cont'd)

(a)



(b)

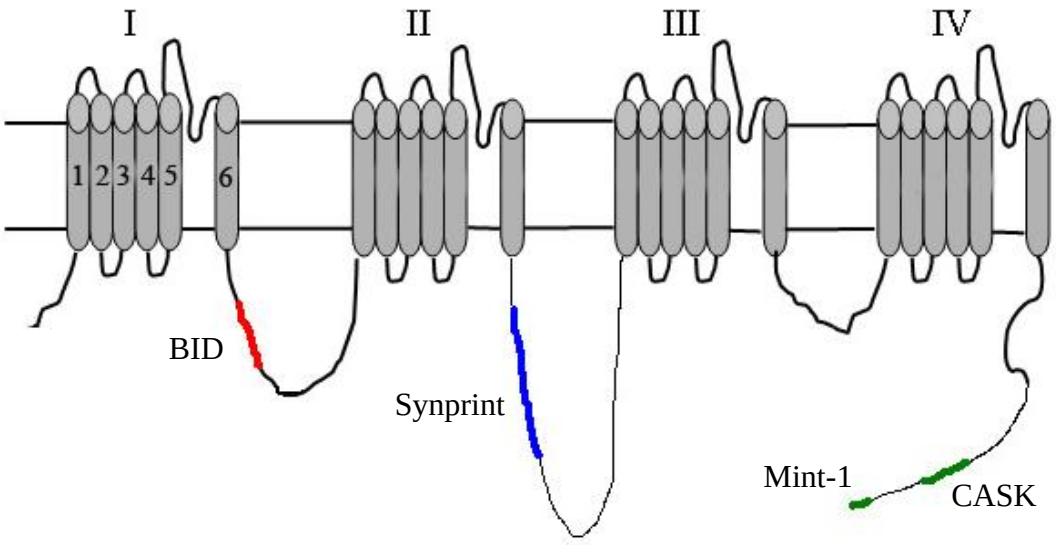


to occur at the synprint (synaptic protein interaction) site of the α_1 subunit, which is regulated by Ca^{2+}. This site has been shown to be necessary for the docking of vesicles via SNAREs to the plasma membrane and subsequent vesicular fusion and transmitter release (Sheng et al., 1991), as well as the interaction site with synaptotagmin (Fig. 1.2).

Different types of VGCCs show differential subcellular distributions in neurons, with N-type and P/Q-type channels located on presynaptic axon terminals, and L-type channels primarily on the cell body and dendrites (Cao et al., 2004; Gomez-Ospina et al., 2006; Li et al., 2004). There is evidence that assembly with different VGCC β isoforms is a factor in determining the subcellular distribution of VGCC (Wittemann et al., 2000). It is, however, now clear that the VGCC α_1 subunit itself is a major determinant of subcellular targeting. Both N- and P/Q-type VGCCs contain a synprint site within the domain II-III linker region that assembles with synaptic proteins such as syntaxin, SNAP-25 and synaptotagmin (Jarvis and Zamponi, 2005). This association is seen to couple synaptic vesicles and the source of extracellular calcium, and serves as a regulatory element by which proteins may control VGCCs function (Jarvis and Zamponi, 2005). This region also appears to play a major role in VGCC targeting. In addition, the C-terminal region of the N-type VGCC contains interaction sites for the adaptor proteins Mint-1 and CASK, both of which are expressed at the nerve terminal (Fig. 1.3) (Zamponi, 2003). This suggests that there must be other factors involved in synaptic targeting. Mochida's group created chimeric VGCCs and showed that the synprint site is involved in synaptic targeting of P/Q-type VGCCs (Mochida et al., 2003). Additionally, Szabo's group, by using naturally occurring domain II-III linker splice variants of human

Figure 1.3: Structure of VGCCs.

The α_1 subunit defines the channel subtype, and is composed of four homologous transmembrane domains, which are connected by large cytoplasmic loops. The domain I-II linker interacts with the β subunit of the VGCCs through the β-interaction domain (BID). In N- and P/Q-type VGCCS, the II-III linker region contains a synaptic protein interaction site (Synprint), which binds syntaxin, SNAP-25 and synaptotagmin. The C-terminus region of the N-type VGCCs contains scaffolding proteins CASK and Mint-1 (Adapted from Spafford and Zamponi, 2003).



N-type VGCCs reported that the presence of an intact synprint site was a crucial determinant of synaptic targeting of N-type VGCCs (Szabo et al., 2006). Furthermore, these authors showed that even N-type channels lacking a synprint region still targeted to axonal compartments rather than remaining in the soma. It appears then, that there are at least two important structural elements in N-type VGCC that are involved in synaptic targeting: the synprint site in the domain II-III linker and an adapter protein interaction site in the C-terminus. These two interaction motifs may be involved at different stages during synaptic vesicle targeting, and the presence of both motifs appears necessary for the channel to reach its presynaptic locus. In the case of P/Q-type VGCCs, the synprint site appears to have a more prominent role, but it is possible that there may be other channel regions, or perhaps preferential association with the β_4 subunit, that may contribute to synaptic targeting. Along these lines, it is worth noting that R-type channels are also located in presynaptic nerve terminals and participate in neurotransmitter release (Kamp et al., 2005) even though they do not have a synprint region, nor Mint-1 and CASK interaction sites in the C-terminus.

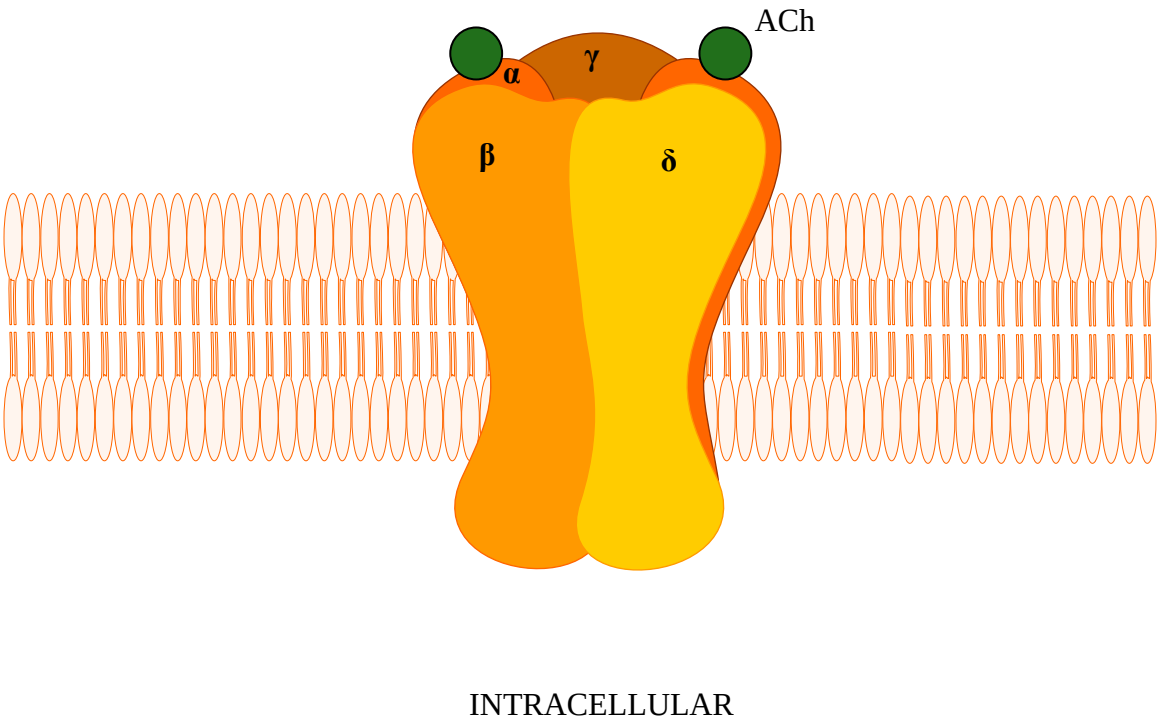
The rab3A protein is also involved in the process of membrane trafficking and release. This is a GTP-binding protein that associates with synaptic vesicles (Fischer von Mollard et al., 1990). In the GTP-bound form, rab3A associates with synaptic vesicle and other proteins (Cao et al., 1998; Shirataki et al., 1993). The association of these proteins may act to fuse and target synaptic vesicles to the membrane (Orci et al., 1998). During or after exocytosis, GTP bound to rab3A is hydrolyzed to GDP leading to the removal of rab3A by guanine nucleotide dissociation inhibitor (Ullrich et al., 1993), thus limiting the fusion of synaptic vesicles with the plasma membrane. Regulation of synaptic vesicle

exocytosis by Ca^{2+} is thought to involve the vesicle protein, synaptotagmin, which acts as the Ca^{2+} sensor (Geppert et al., 1994; Li et al., 1995a; Li et al., 1995b). Synaptotagmin has two Ca^{2+} binding regions on its cytoplasmic side that are homologous to a region within protein kinase C (Davletov and Südhof, 1993; Perin et al., 1990). It is thought that binding of Ca^{2+} induces electrostatic changes of synaptotagmin to facilitate association with syntaxin and phospholipids leading to rapid exocytosis (Shao et al., 1998; Ubach et al., 1998).

Once synaptic vesicles fuse with the nerve terminal membrane, ACh is released and diffuses across the synaptic cleft where it binds specifically to post-synaptic ACh receptors localized in the end-plate region of the muscle (Landau, 1978). The nACh receptor is a large pentamer complex arranged in a ring around a central ion pore (Fig. 1.4). There are two α subunits, and a β, a γ, and a δ subunit (Ashcroft, 2000). The ACh binding sites are on the α subunits, and full activation of the channel requires that both sites be occupied. When two ACh molecules bind to an ACh receptor, a conformational change in the receptor opens the central pore and allows cations to flow into or out of the muscle cell (Takeuchi, 1963). Since generally more Na^{+} flows in than K^{+} leaves (due to electrochemical gradients), the muscle cell is depolarized (Boyd and Martin, 1956; Fatt and Katz, 1951). If enough ACh is released, then sufficient ACh receptor channels are activated and the muscle cell membrane is depolarized to threshold for opening voltage-gated Na^{+} channels nearby and initiating a muscle action potential. Generally, stimulation of a motoneuron automatically releases enough ACh into the cleft to

Figure 1.4: Structure of the nicotinic acetylcholine (nACh) receptor.

The nACh receptor contains a β, γ and δ subunits as well as two α subunits which contain the ACh binding site. (Adapted from Levitan and Kaczmarek, 1997).



stimulate the adjacent muscle cell to threshold. Thus, an action potential (with ion fluxes similar to the neuronal action potential) is initiated at the postsynaptic site on the muscle cell membrane. The muscle action potential then activates a cascade of events that leads to muscle contraction.

The activation of the ACh receptors comes to an end when the enzyme acetylcholinesterase (AChE) hydrolyzes ACh to acetate and choline (Fig. 1.1) (Gaspersic et al., 1999; Rotundo et al., 1998). The AChE is found in high concentrations in the synaptic cleft. Due to its high concentration and fast catalytic rate (10^4 to 10^5 substrate molecules hydrolyzed per second), the concentration of ACh in the synaptic cleft drops very quickly following its release. Choline is then taken up into the nerve terminal via a high-affinity sodium-dependent choline up-take system and reused to synthesize new ACh molecules which are then packaged into synaptic vesicles for posterior use (Marchbanks, 1982).

C. Voltage – Gated Calcium Channels

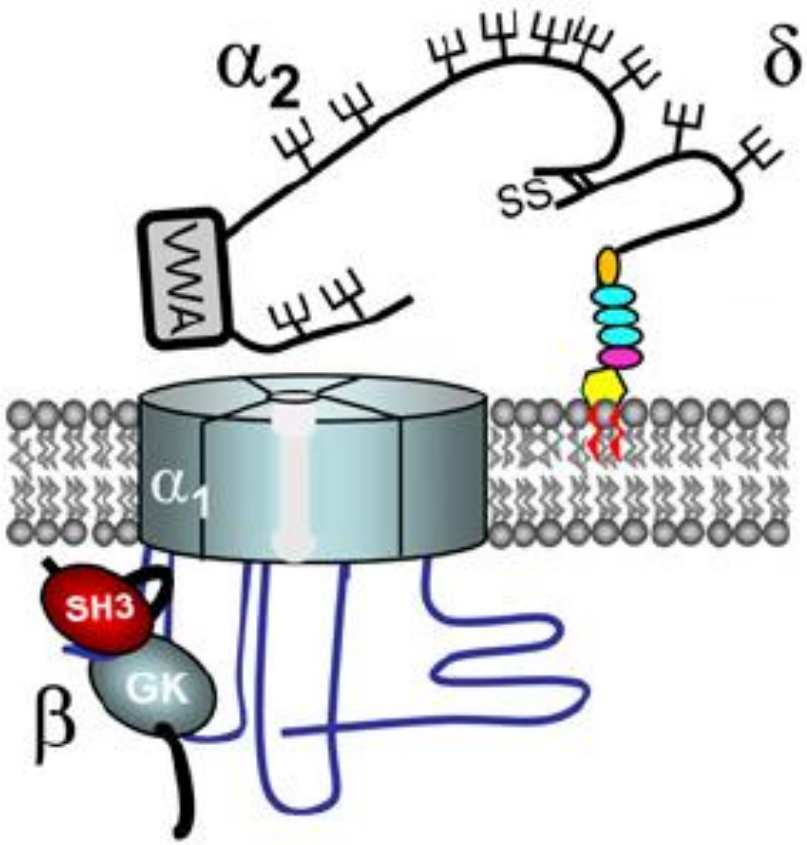
1. General Description

VGCCs are expressed in the plasma membrane of virtually all excitable cells that transduce electrical activity into intracellular biochemical signals. At resting membrane potential, these channels are normally closed, but they become activated (open) at depolarized membrane potentials. When channels are activated, Ca^{2+} enters the cell, acting as a second messenger that plays vital roles in cellular metabolism, excitability, contraction, gene regulation, hormonal and neurotransmitter release, depending on the cell type (Augustine et al., 1987; Miller, 1987). Since Ca^{2+} plays so many important roles

in various physiological functions, a slight alteration of the intracellular Ca^{2+} homeostasis will produce a wide array of phenotypes and disorders. The importance of Ca^{2+} in cellular functions requires precise spatial and temporal control, and as such, various mechanisms exist to control Ca^{2+} levels within the cell. One of these is the gating of VGCCs. The existence of multiple types of VGCCs, each with distinct biophysical properties, distribution, and densities on cell membranes, allows precise control of Ca^{2+} entry into the cell. Another important difference between VGCCs is their sensitivity to depolarization. Some channels activate with small depolarizations, and therefore belong to the class of low voltage-activated (LVA) channels. These types of channels usually have rapid, voltage-dependent inactivation. The other class is the high voltage-activated (HVA) channels, which require large depolarizations to activate. These kinds of channels often lack rapid inactivation, and therefore their activity can be recorded in isolation from LVA currents starting from depolarized holding potentials. Later studies, further classified Ca^{2+} currents into L, N, P, Q, R, and T based upon their molecular, biochemical, pharmacological, and electrophysiological characteristics (Table 1.1) (Catterall, 2000; Randall and Tsien, 1995; Snutch et al., 1990; Tsien et al., 1988; Zhang et al., 1993). Modern nomenclature refers to them as Ca_v 1.1-1.4 (L-type), Ca_v 2.1-2.3 (P/Q-, N- and R-type), and Ca_v 3.1-3.3 (T-type). The Ca_v 2 is specifically found in neurons.

Figure 1.5: Molecular organization of VGCCs.

Subunit composition of HVA VGCCs. The α_1 subunit is a transmembrane protein composed of four domains. The $\alpha_2\delta$ subunit showing the glycosylphosphatidylinositol (GPI) anchor consists of ethanolamine (orange), three mannose rings (blue), glucosamine (pink), and inositol (yellow). The β subunit is located in the cytoplasm, and interacts with the α_1 subunit. Some VGCCs may have an additional transmembrane protein, the γ subunit. (Davies et al., 2010).



The VGCCs are composed of five subunits: α_1, α_2, β, δ, and γ. α_2 and δ are linked posttranslationally by disulfide bonds into a single subunit referred to as $\alpha_2\delta$ (Takahashi et al., 1987). L-, N-, P/Q- and R-type channels are made up of α_1, $\alpha_2\delta$, β, and in some tissues γ subunits (Fig. 1.5). T-type channels, on the other hand, appear to require only an α_1 subunit (Perez-Reyes, 2003; 2006).

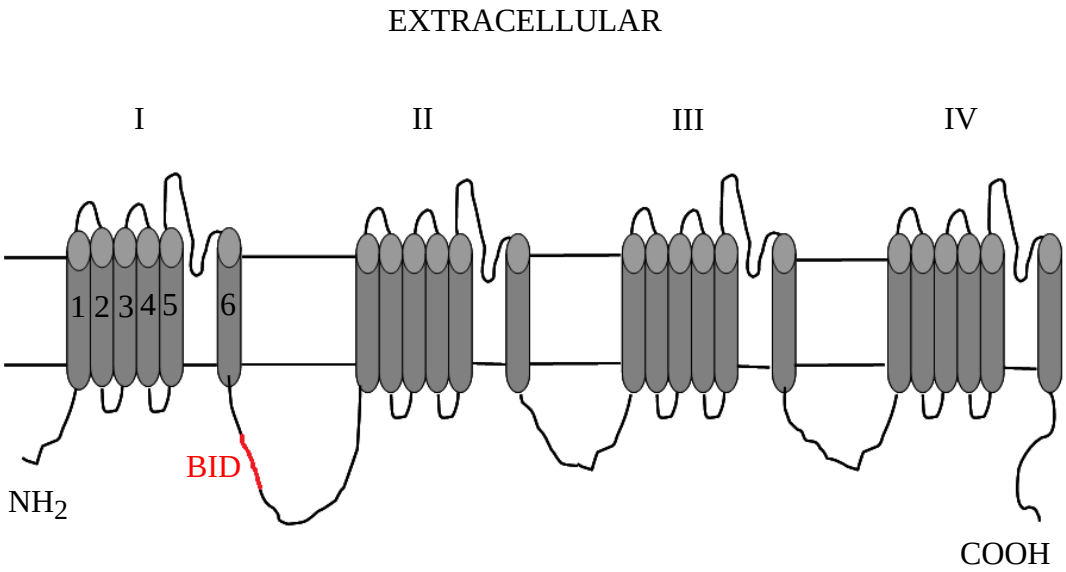
The α_1 subunit is responsible for their unique biophysical and pharmacological properties. However, proper trafficking and functioning of L-, N-, P/Q- and R-type channels require the presence of the auxiliary subunits. In particular, the β subunit plays a very important role in trafficking the channels to the plasma membrane, fine-tuning channel gating and regulating channel modulation by other proteins and signaling molecules.

2. The α_1 Subunit

The α_1 subunit is the principal subunit of VGCCs. It is a 190-250 kDa protein containing four homologous repeats (I-IV) connected through cytoplasmic loops (Fig. 1.6). Each repeat has six predicted transmembrane segments (S1-S6) and a reentrant pore forming loop (P-loop) between S5 and S6. The four P-loops form the ion-selectivity filter, where four highly conserved negatively charged amino acids (glutamate or aspartate), one from each P-loop, form a signature locus that is essential for selecting and conducting Ca^{2+} (Kim et al., 1993; Kuo and Hess, 1993; Sather and McCleskey, 2003; Yang et al., 1993). The S6 segments form the inner pore (Zhen et al., 2005), and the S4

Figure 1.6: Stucture of the α_1 subunit.

Schematic representation of the predicted transmembrane topology of the α_1 subunit, with the localization of the β-interaction domain (BID) marked in red (Adapted from Burgess et al., 1997).



segments' positively charged amino acids form part of the voltage sensor. The voltage-dependent movement of this sensor results in channel opening and closing. Furthermore, the majority of drug and toxin binding sites are located on the α_1 subunit (Catterall, 2000). Thus, the α_1 subunit possesses all the key features that define a VGCC, including pharmacological and biophysical properties such as gating, ion selectivity, and permeation.

Mammalian α_1 subunits are encoded by 10 distinct genes (Table 1.2). Based on amino acid sequence similarity, the α_1 subunit is divided into three subfamilies: Ca_V1, Ca_V2, and Ca_V3 (Arikkath and Campbell, 2003; Catterall, 2000; Ertel et al. 2000; Yang and Berggren, 2006). The Ca_V1 subfamily includes channels that conduct L-type Ca^{2+} currents; the Ca_V2 subfamily includes channels that conduct N-, P/Q-, and R-type Ca^{2+} currents; and the Ca_V3 subfamily includes channels that conduct T-type Ca^{2+} currents.

The Ca_V1 subfamily, or L-type, is composed of α_{1C}, α_{1D}, α_{1F} or α_{1S}. They belong to the HVA subfamily of VGCCs. They are located mainly in skeletal, smooth (Almers et al., 1981; Rosenberg et. al., 1986; Sanchez and Stefani, 1978), and cardiac muscle (Reuter, 1983; Tsien, 1983), endocrine cells where they initiate hormone release (Milani et al., 1990), and in neurons where they are important in regulation of gene expression and in integration of synaptic inputs (Bean, 1989; Llinas et al., 1992; Tsien et al., 1988; Zhang et al., 1993). L-type VGCCs replenish cellular Ca^{2+} during periods of rapid activity and increase intracellular calcium in response to tetanic stimulation, which

Table 1.1: Pharmacological and biophysical properties of VGCC subtypes

(Adapted from Hille, 2000; Urbano et al., 2008).

	HVA	**HVA**	**LVA**
Ca^{2+} current type	L	P/Q, N, R	T
Structural nomenclature	Ca_V 1.1, 1.2, 1.3, 1.4	Ca_V 2.1, 2.2, 2.3	Ca_V 3.1, 3.2, 3.3
α_1 subunits	α_{1C}, α_{1D}, α_{1F}, α_{1S}	α_{1A}, α_{1B}, α_{1E}	α_{1G}, α_{1H}, α_{1I}
Activation range	Positive to -10 mV	Positive to -20 mV	Positive to -70 mV
Inactivation range	-60 to -10 mV	-120 to -30 mV	-100 to -60 mV
Inactivation	Very slow (τ > 500 ms)	Partial (τ ~ 50 – 80 ms)	Complete (τ ~ 20 – 50 ms)
Deactivation rate	Rapid	Slow	Rapid
Single-channel conductance	25 pS	13 pS	8 pS
Single-channel openings	Continual reopening	Long burst	Brief burst, Inactivation
Relative conductance	$Ba^{2+} > Ca^{2+}$	$Ba^{2+} > Ca^{2+}$	$Ba^{2+} = Ca^{2+}$
ω-conotoxin GVIA	Resistant	Ca_V2.2 sensitive	Resistant
Dihydropyridines	Sensitive	Resistant	Resistant
ω-agatoxin IVA	Resistant	Ca_V2.1 sensitive	Resistant
Calciseptine	Sensitive	Resistant	Resistant
SNX-482	Resistant	Ca_V2.3 sensitive	Resistant
Divalent block	$Cd^{2+} > Ni^{2+}$	$Cd^{2+} > Ni^{2+}$	$Ni^{2+} > Cd^{2+}$

leads to increased contractile force. The main physiological role for skeletal muscle VGCCs is to serve as a voltage sensor in excitation-contraction coupling in cardiac, skeletal, and smooth muscles (Bean, 1989). VGCCs in the transverse tubule membranes are thought to interact physically with the calcium release channels located in the sarcoplasmic reticulum membrane. Voltage-driven conformational changes in VGCCs then activate calcium release from the sarcoplasmic reticulum via ryanodine receptor-mediated stores (Adams and Beam, 1990; Catterall, 1991; Rios and Brum, 1987).

L-type VGCCs mediate "long-lasting" currents when Ba^{2+} is the current carrier (this characteristic gave rise to the channel's name) (Nowycky et al., 1985). With slow voltage-dependent inactivation, they have a large single-channel conductance (~ 25 pS) and a high voltage of activation, and they are specifically inhibited by the organic dihydropyridine compounds (Catterall and Striessnig, 1992; Hofmann et al., 1994). L-type VGCCs are also antagonized by the peptide toxin, calciseptine, isolated from the black mamba snake, *Dendroaspis polylepsis polylepsis*, as well as the antihypertensive drugs diltiazem and verapamil.

There are several disorders associated with mutations in L-type channels (Table 1.2). The disorder type varies according to which α_1 subunit is mutated. Mutations in the human α_{1C} subunit (CACNA1C) result in Timothy's Syndrome, a multi-system disorder including syndactyly (a condition in which two or more digits are fused together), immune deficiency and long QT interval (the QT interval represents electrical depolarization and repolarization of the left and right ventricles. A prolonged QT interval is a biomarker for ventricular tachyarrhythmias and a risk factor for sudden death) and

Table 1.2: Human and mouse disorders associated with VGCCs subunits

(Adapted from Burgess et al., 1999; Mckeown et al., 2006)

Subunit	Channel type	Human gene	Associated disorder (mouse and human)
α_{1A}	P/Q	CACNA1A	Humans: Episodic ataxia type-2; familial hemiplegic migraine; spinal cerebellar ataxia type-6, sporadic hemiplegic migraine with ataxia and nystagmus. Mice: *tg, tg^{la}, tg^{rol}, tg^{4J}, and tg^{5J}*
α_{1B}	N	CACNA1B	Humans: Mutations in this gene have not been reported. $\alpha_{1B}^{-/-}$ mice: problems in nociception, decrease in sympathetic nervous system function and alterations in response to ethanol and anesthetics.
α_{1C}	L	CACNA1C	Humans: Timothy syndrome. Mice: knockout is lethal due to cardiac dysfunction.
α_{1D}	L	CACNA1D	Humans: Ca_V 1.3 has been postulated to be related to Parkinson's disease, though not necessarily due to a mutation. Mice: congenital deafness.
α_{1E}	R	CACNA1E	Humans: no mutations have been associated with this gene. Mice: mutations in this gene affect glucose-stimulated insulin release from pancreatic β cells by facilitating the entry of calcium needed for granule replenishment.
α_{1F}	L	CACNA1F	Humans: X-linked congenital stationary blindness type 2.
α_{1G}	T	CACNA1G	Humans: no mutations have been associated with this gene. Mice: reduced sleep patterns, bradycardia and delayed atriventricular conduction.

Table 1.2 (cont'd)

Subunit	Channel type	Human gene	Associated disorder (mouse and human)
α_{1H}	T	CACNA1H	Humans: childhood *absence* epilepsy, idiopathic generalized epilepsy type 6, autism spectrum disorders.
α_{1I}	T	CACNA1I	Humans: No mutations have been associated with this gene.
α_{1S}	L	CACNA1S	Humans: hypokalemic periodic paralysis, malignant hyperthermia susceptibility. Mice: Muscular dysgenesis (lethal).
β_1	n.a.	CACNB1	
β_2	n.a	CACNB2	
β_3	n.a	CACNB3	
β_4	P/Q	CACNB4	Humans: episodic ataxia, juvenile myoclonic epilepsy, generalized epilepsy. Mice: *lethargic*
$\alpha_2\delta_1$	n.a.	CACNA2D1	Humans: malignant hyperthermia
$\alpha_2\delta_2$	n.a.	CACNA2D2	Mice: *ducky*, they present spike-wave seizures and ataxia.
$\alpha_2\delta_3$	n.a.	CACNA2D3	
$\alpha_2\delta_4$	n.a.	CACNA2D4	Humans: retinal cone dystrophy 4
γ_1	n.a.	CACNG1	

Table 1.2 (cont'd)

Subunit	Channel type	Human gene	Associated disorder (mouse and human)
γ_2	n.a.	CACNG2	Mice: *stargazer*, they present ataxic gait, paroxysmal dyskinesia, frequent spike–wave discharges, characteristic of absence seizures in humans.
γ_3	n.a.	CACNG3	
γ_4	n.a.	CACNG4	
γ_5	n.a.	CACNG5	
γ_6	n.a.	CACNG6	
γ_7	n.a.	CACNG7	
γ_8	n.a.	CACNG8	

ventricular arrhythmias during infancy (Splawski et al., 2004; Splawski et al., 2005). Another subunit present in L-type VGCCs is the α_{1F} (CACNA1F) subunit in which several mutations have been identified in patients with incomplete X-linked congenital stationary night blindness (Miyake et al., 1986; Tremblay et al., 1995), as well as in families with X-linked cone dystrophy (Jalkanen et al., 2006). On the other hand, missense mutations in the CACNA1S gene have been associated with human cases of hypokalemic periodic paralysis and malignant hyperthermia susceptibility (Elbaz et al., 1995; Jurkat-Rott et al., 1994; Wang et al., 2005).

Using the skeletal muscle α_1 subunit as a probe, five additional genes encoding α_1 subunits of VGCC have been identified by cDNA cloning and sequencing (Snutch and Reiner, 1992; Soong et al., 1993; Zhang et al., 1993). The α_1 subunits fall into two groups based on amino acid sequence similarity, the L-type and the non-L type. The class C and D genes encode the L-type in which sequences are greater than 75% identical to skeletal muscle L-type α_1 subunits. The class C gene is mainly present in calcium channels localized in the heart and is widely expressed in other tissues. The class D gene is expressed in neuroendocrine cells and neurons. Class A, B, and E genes encode non-L type channel, expressed primarily in neurons, in which the amino acid sequences are only 25 to 40% identical to the skeletal muscle α subunits. In general, the level of amino acid sequence identity among the α_1 subunits is greatest in the transmembrane regions and least in the large intracellular loops connecting domains I, II, and III and in the

intracellular amino-terminal and carboxy terminal domains. Most of the α_1 subunit genes also encode alternatively spliced segments that increase their molecular diversity.

The non-L-type calcium channels (Ca_v 2.x) contain α_{1A} (which forms part of the P/Q-type), α_{1B} (N-type) or α_{1E} (R-type), all of which are distinct from the L-type VGCC and belong to the Ca_v2 subfamily. All these channels are present in neurons, and have intermediate single channel conductances (~ 15 pS) and can mediate Ca^{2+} currents with varying rates of voltage-dependent inactivation, depending on their subunit composition and other factors. They are best distinguished by their pharmacological properties.

P-type Ca^{2+} current was initially identified in Purkinje cells, and is blocked at low concentrations by the spider venom ω-agatoxin IVA (Aga-IVA) (Katz et al., 1995; Mintz et al., 1992). An Aga-IVA sensitive current also was found in cerebellar granule cells. However, this Ca^{2+} current exhibits properties resembling those described using cloned P-like channel proteins and were named Q-type channels (Randall and Tsien, 1995; Wheeler et al., 1994; Zhang et al., 1993). They are found throughout the nervous system controlling fast neurotransmitter release, mainly at excitatory synapses.

Recessive mutations in the α_{1A} subunit in mice lead to the *tottering* phenotype (Table 1.2) which is characterized by ataxia, polyspike discharges, and intermittent dystonic episodes. Mutations in the *tottering* locus disrupt the *Cacna1a* gene, which encodes the α_{1A} subunit of P/Q-type (Doyle et al., 1997; Fletcher et al., 1996). There are five alleles at the tottering locus, which include: *totttering* (*tg*), *tottering leaner* (tg^{la}),

tottering rolling Nagoya (tg^{rol}), *tottering 4 Jackson* (tg^{4J}), and *tottering 5 Jackson* (tg^{5J}) (Green and Sidman, 1962; Lorenzon et al., 1998; Oda, 1981; Tsuji and Meier, 1971). Mice homozygous for the original allele, *tg*, exhibit three distinct, recessively inherited neurological phenotypes: epilepsy, ataxia, and episodic (or paroxysomal) dyskinesia. These phenotypes become apparent at 3 weeks of age and continue throughout life. However, it was recently shown that the N- and R- type VGCCs control ACh release at the adult NMJ (Pardo et al., 2006). R-type VGCCs also control ACh release at some enteric synapses (Naidoo et al., 2010). In humans, mutations in the CACNA1A gene give rise to three distinct neurological disorders: episodic ataxia type-2, familial hemiplegic migraine type 1, spinal cerebellar ataxia type 6, and certain cases of *absence* epilepsy (Bidaud et al., 2006; Jeng et al., 2006; Ophoff et al., 1996; Pietrobon, 2005; Zhuchenko, et al., 1997). Moreover, the α_{1A} subunit is the main target for the attack of autoantibodies of Lambert Eaton myasthenic syndrome (LEMS), an autoimmune disorder. LEMS is a neuromuscular disorder characterized by reduced ACh release, due to autoantibodies directed against VGCCs, mainly the P/Q-type (Flink and Atchison, 2003; Pinto et al., 2002). These disorders illustrate the extensive diversity of phenotype that can arise from alterations in a single VGCC subunit isoform.

The R-type ($Ca_V2.3$) was observed to be resistant to the action of all the VGCC antagonists, however it was sensitive to SNX-482, a synthetic peptide whose structure was based on a peptide derived from the venom of the tarantula *Hysterocarates gigas*. This channel is mainly localized in the somata and dendrites of central neurons, as well as nerve terminals of central synapses (Breustedt et al., 2003; Kamp et al., 2005). No human

mutations have been identified with $Ca_v2.3$ (Table 1.2). However, studies done in knockout mice (Jing et al., 2005) show that it affects glucose-stimulated insulin release from pancreatic β cells by facilitating the global entry of calcium needed for granule replenishment.

The N-type VGCCs are specifically inhibited by ω-conotoxin GVIA (Ctx GVIA), which is derived from the *Conus geographus* snail (Grantham et al., 1994; Hillyard et al., 1992; McCleskey et al., 1987; Tsien et al., 1988).They are also non-selectively inhibited by ω-conotoxin MVIIC (Cntx MVIIC) which is derived from the *Conus magus* snail. They have intermediate voltage-dependence and rate of inactivation: more negative and faster than L-type, but more positive and slower than T-type (Fox et al., 1987a,b; Nowycky et al., 1985). This gave rise to its name, since its current was neither L- nor T-type. There are no reports of mutations in the CACN1B gene in the human population (Table 1.2). Studies done in $\beta_{1B}^{-/-}$ knockout mice show nociception problems (Saegusa et al., 2002), decreased sympathetic nervous system function (Ino et al., 2001) and alterations in response to ethanol (Newton et al., 2004) and anesthetics (Takei et al., 2003). N-type VGCCs have been shown to play a prominent role in neuropathic pain in humans and are the target of a drug derived from the ω-conotoxins such as Ziconotide® (Berecki et al., 2010; Lee et al., 2010; Schmidtko et al., 2010).

The T-type VGCCs, belong to the Ca_v3 subfamily, and are a subtype of LVA class VGCCs. They were designated T-type because of their transient kinetics and tiny conductance. In comparison to L-type, these Ca^{2+} currents activate at much more negative membrane potentials, inactivate and deactivate rapidly, have small single

channel conductance, and they are insensitive to VGCC antagonists (Carbone and Lux, 1984; Nowycky et al., 1985). People with mutations in the CACNA1H gene can suffer from idiopathic generalized epilepsy, childhood *absence* epilepsy (Chen et al., 2003; Robinson and Gardiner, 2000) or autism spectrum disorders (Splawski et al., 2006) (Table 1.2).

3. The $\alpha_2\delta$ Subunit

The Ca_v1 and Ca_v2 subfamilies contain an auxiliary $\alpha_2\delta$ subunit (Davies et al., 2007). To date there are four known $\alpha_2\delta$ subunits ($\alpha_2\delta_1$ - $\alpha_2\delta_4$), each encoded by a unique gene and all possessing splice variants. Each $\alpha_2\delta$ protein is encoded by a single messenger RNA (mRNA) and is cleaved posttranslationally into α_2 (extracellular) and δ (transmembrane) subunits. These are then linked by disulfide bonds (Klugbauer et al., 1999; Klugbauer et al., 2003; Qin et al., 2002). The δ peptide, originally presumed to be transmembrane but recently shown to be attached to the membrane through a glycosylphosphatidylinositol linker (Davies et al., 2010), anchors the larger extracellular α_2 peptide in place (Fig. 1.5). $\alpha_2\delta$ subunits can modify channel biophysical properties (Canti et al., 2003; Singer et al., 1991; Wakamori et al., 1994), but their main role is to increase Ca^{2+} current (Canti et al., 2003; Davies et al., 2006; Gao et al., 2000; Klugbauer et al., 1999; Klugbauer et al., 2003; Singer et al., 1991; Wakamori et al., 1994) by promoting trafficking of the α_1 subunit to the plasma membrane and/or by increasing its retention there (Bernstein and Jones, 2007; Canti et al., 2005; Gurnett et al., 1997;

Sandoval et al., 2004). More recently, it was reported that $\alpha_2\delta$ functioned as a thrombospondin receptor to regulate excitatory synaptogenesis, independently from its regulation of VGCC activity (Eroglu et al., 2009; Kurshan et al., 2009).

The *ducky* phenotype results from a naturally occurring mutation caused by the loss of the $\alpha_2\delta_2$ protein (Table 1.2). It is characterized by shortened life span, *absence* epilepsy, spike wave seizures, cerebellar ataxia, decreased whole cell P/Q-type current densities, and decreased Purkinje cell dendritic arborization and firing rates (Barclay et al., 2001; Davies et al., 2007; Donato et al., 2006; Klugbauer et al., 2003). $\alpha_2\delta_2$ knockouts also have abnormalities in the cardiovascular, immune, respiratory, and nervous systems. Irregularities in the cardiovascular system are also found in $\alpha_2\delta_1$ knockouts (Fuller-Bicer et al., 2009). $\alpha_2\delta_3$-null *Drosophila* are not viable, and the mutants have significantly impaired synaptic transmission (Dickman et al., 2008; Kurshan et al., 2009). Upregulation of $\alpha_2\delta_1$, on the other hand, is associated with neuropathic pain (Li et al., 2004; Li et al., 2006). In humans, linkage analysis has indicated that mutations in the $\alpha_2\delta_1$ subunit may play a role in malignant hyperthermia susceptibility, although no mutations have yet been found in the CACNL2A gene that encodes for the $\alpha_2\delta_1$ subunit (Illes et al., 1994). The $\alpha_2\delta_1$ subunit is the target of a pharmacological agent (Gabapentin) used to treat intractable pain; the mechanism by which this reduces nociception is unknown (Field et al., 2007**;** Hendrich et al., 2008; Kusunose et al., 2010**;** Van Elstraete et al., 2008)**.**

4. The γ Subunit

There are eight different γ subunit genes (γ_{1-8}), all yielding proteins with four transmembrane segments flanked by cytoplasmic N- and C- termini. This subunit increases the current densities (Yasuda et al., 2004), and alters pharmacological and functional properties of the channels. γ_1 was the first cloned γ subunit (Glossmann et al., 1987; Jay et al., 1990; Takahashi et al., 1987) and was copurified with muscle VGCCs, consistent with its primary role as a VGCC subunit. γ_1 knockout mice are viable, morphologically indistinguishable from *wt* mice, but have larger Ca^{2+} currents with altered inactivation kinetics (Freise et al., 2000). γ_2, γ_3, and γ_4 also associate with VGCCs (Kang et al., 2001; Sharp et al., 2001). γ_{1-4} subunits have been shown to produce varying effects on VGCC activity, depending on the partnered α_1 and β subunit (Eberst et al., 1997; Freise et al., 2000; Held et al., 2002; Kang et al., 2001; Klugbauer et al., 2000; Letts et al., 1998; Rousset et al., 2001; Singer et al., 1991; Wei et al., 1991). The most consistent effect is a small reduction of current, caused mainly by a hyperpolarizing shift of inactivation voltage and/or a positive shift of activation voltage. The γ_2, γ_3, γ_4, and γ_8 regulate the trafficking, localization, and biophysical properties of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors (Black, 2003; Chen et al., 2007; Kang and Campbell, 2003; Osten and Stern-Bach, 2006; Tomita et al., 2004). They are, therefore, referred to as transmembrane AMPA receptor regulatory proteins (TARPs). Indeed, acting as TARPs seems to be the primary role of γ_2, γ_3, γ_4, γ_8, and probably of γ_7

(Kato et al., 2007). While the function of γ_5 remains unknown, γ_6 is suggested to inhibit $Ca_v3.1$ channels (Lin et al., 2008), and γ_7 is involved in the turnover of the mRNA of $Ca_v2.2$ and other proteins (Ferron et al., 2008; Moss et al., 2002). Mutations in γ_2 underlie the *stargazer* mouse phenotype (Letts et al., 1998), which includes early onset ataxia, spike-wave seizures, *absence* epilepsy (Noebels et al., 1990), and defects in the cerebellum and inner ear.

5. The β Subunit

Purified Ca_v1 and Ca_v2 channels contain a tightly bound cytosolic β protein (Fig. 1.5). There are four subfamiles of β (β_{1-4}), each with splice variants, encoded by four distinct genes, *Cacnb 1-4*. They all have 14 exons except *Cacnb3*, which has 13; each β subunit has 2 or more splice variants. The β subunits are abundantly expressed in excitable tissues such as brain, heart, and muscles. The expression of some variants is developmentally regulated. For example, β_{1b} and β_4 expression increases with development (McEnery et al., 1998; Pichler et al., 1997; Witcher et al., 1995), whereas β_{2c}, β_{2d} and β_{2e} expression is decreased in adults (Chu et al., 2004; Herzig et al., 2007; Hullin et al., 1992).

All four β subunits can dramatically enhance Ca^{2+} channel currents when they are coexpressed in heterologous expression systems along with a Ca_v1 or Ca_v2 α_1 subunit (Lacerda et al., 1991; Mikami et al., 1989; Mori et al., 1991; Pragnell et al., 1994; Shistik

et al., 1995; Varadi et al., 1991; Wei et al., 1991; Williams et al., 1992). Since the association between the β subunit and α_1 subunit is promiscuous (i.e., any full length β subunit can associate with any Ca_V1 or Ca_V2 α_1 subunit), alternative splicing greatly increases the molecular diversity and functionality of HVA Ca^{2+} channels. β subunits change the voltage-dependence and kinetics of activation and inactivation (Josephson and Varadi, 1996; Lacerda et al., 1991; Mori et al., 1991; Singer et al., 1991; Stea et al., 1993; Stephens et al., 2000; Varadi et al., 1991). However, they do not affect ion permeability (Gollasch et al., 1996; Shistik et al., 1995; Wakamori et al., 1993). The β subunits antagonize endoplasmic reticulum (ER) retention of the α_1 subunit. For P/Q-type channels it has been proposed that the α_1 subunit may contain multiple ER retention motifs that are masked upon β subunit binding (Geib et al., 2002), thus allowing for cotrafficking of the α_1-β subunit protein complex to the plasma membrane. The β subunits have multiple consensus sites for phosphorylation. Co-expression of different β subunits may induce different regulation by protein phosphorylation/dephosphorylation mechanisms. Furthermore, the β subunit either regulates or is indispensable for the modulation of Ca_V1 and Ca_V2 channels by protein kinases, G proteins, and small RGK proteins. The β subunit can lower the energy barrier for opening the channel pore and thereby increase peak Ca^{2+} currents without increasing the number of VGCCs expressed. In the case of β_1 and β_2, knockout to these subunits results in the animals being nonviable (Chien et al., 1995; Gregg et al., 1996; Strube et al., 1996; Strube et al., 1998);

in the case of β_3 and β_4 it results in severe phenotype (Burgess el at., 1999; McEnery et al., 1998; Murakami et al., 2003a,b; Namkung et al., 1998).

In 1989, Ruth's group successfully cloned the first β subunit using a classical approach based on peptide sequences derived from a purified skeletal muscle β subunit (Ruth et al., 1989). This β subunit is now referred to as β_{1a}. Later, using a labeled skeletal muscle β_{1a} cDNA, another group screened a rat brain cDNA library (Pragnell et al., 1991) and cloned a new β subunit, which was a splice variant of β_1, and was named β_{1b} (Powers et al., 1992).

Using a rat brain cDNA library with β_{1a}, and low-stringency hybridization, β_{2a} was discovered (Perez-Reyes et al., 1992). While using a cardiac cDNA library, β_{2a}, two other splice variants (β_{2b} and β_{2c}) were found, and cDNA for β_3 was isolated (Hullin et al., 1992). β_3 and β_4 subunits were cloned from a rat brain cDNA library, using degenerate primers corresponding to the conserved domains of β_1 and β_2 (Castellano et al., 1993).

All β subunits are expressed in the brain. However, β_1 is the only one that is also expressed in skeletal muscle. The importance of this subunit is demonstrated by mutations in the β_1 subunit (Table 1.2), which result in the loss of excitation contraction coupling in skeletal muscle, and is lethal (Gregg et al., 1996; Strube et al., 1998).

Mutations in the β_3 subunit lead to altered kinetics in dorsal root ganglia and sympathetic cervical ganglion neurons (Murakami et al., 2003; Namkung et al., 1998).

The β_4 subunit, which normally associates with the α_{1A} subunit of the P/Q-type VGCCs, is widely expressed in the brain. It has been reported that spontaneous mutations in this subunit cause several neurological syndromes in mice (Burgess et al, 1997), producing effects on VGCC expression and function. These involve reduction in VGCC targeting, assembly, membrane insertion, channel density and alteration in kinetic parameters, vesicular release and synaptic transmission. Also, the loss of a functional β_4 subunit can impact the function of α_{1A} – containing calcium channels.

The β_4 subunit has the ability to interact with any one of at least four neuronal calcium channel α_1 subunits in addition to its preferred partner, α_{1A}. In *wt* mouse brain, α_{1A} preferentially pairs with β_4 subunit. While preferential α_1/β pairings ($\alpha_1 + \beta_4 > \beta_3 >> \beta_{1b} \geq \beta_2$) are inferred from binding studies of *in vitro* translated subunits (Liu et al., 1996); coimmunoprecipitation experiments demonstrate that each of the four known β subunits can associate with native L (α_{1C}, α_{1D}), N (α_{1B}) and P/Q-type (α_{1A}), channels in brain (Liu et al., 1996; Pichler et al., 1997; Scott et al., 1996). They are thus likely to regulate more than a single α_1 subtype. Other experiments show that heterogeneous α_1-β pairings can occur within a presumably homogeneous population of cultures of PC12 cells (Liu et al., 1996). Thus, while mutations in α_1 subunit genes affect the function of a single specific channel type, mutation of a β subunit can simultaneously modify multiple

channel types, and independently alter Ca^{2+} current properties at various sites and developmental stages in the CNS.

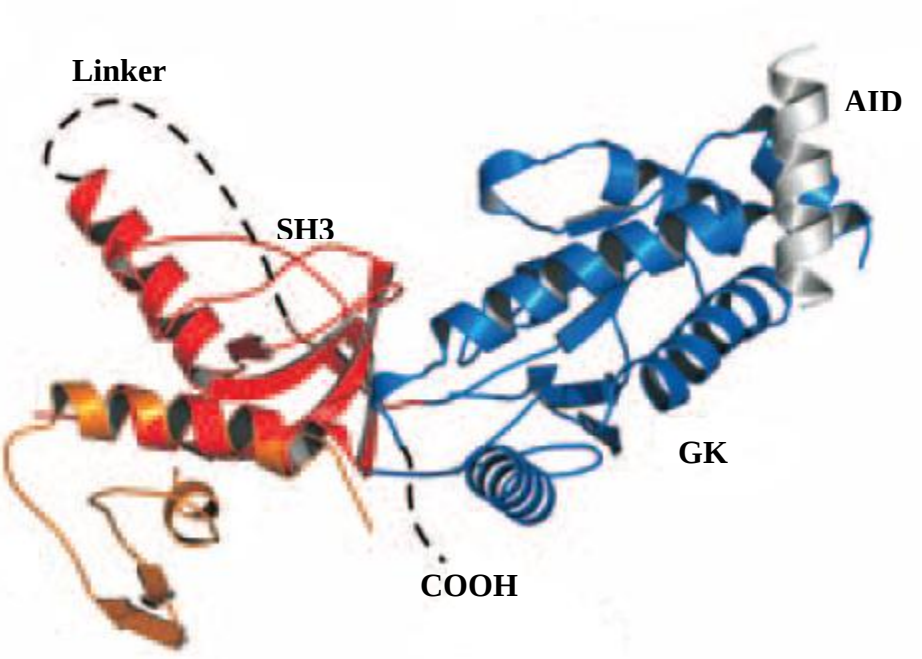
It has been shown that truncation and missense mutations in CACNB4 gene are associated with families having juvenile myoclonic epilepsy, generalized epilepsy and episodic ataxia (Escayg et al., 2000) (Table 1.2).

5.1 Structure of the β subunit

The β subunit has a modular structure consisting of five distinct regions (Birnbaumer et al., 1998; Colecraft et al., 2002; De Waard et al., 1994; Hanlon et al., 1999; Opatowsky et al., 2003; Pragnell et al., 1994). The first, third, and fifth regions are variable in length and amino acid sequence, whereas the second and fourth regions are highly conserved and are more homologous to the Src homology 3 (SH3) and guanylate kinase (GK) domains, respectively. The SH3 domain is a common protein interaction module present in diverse groups of proteins. The GK domain is also engaged in protein-protein interactions (Elias and Nicoll, 2007; Funke et al., 2005; Takahashi et al., 2004). The middle three regions of the β subunit constitute the β subunit core, which is able to reconstitute many key functions of the β subunit (Chen et al., 2004; Chen et al., 2009; De Waard et al., 1994; Gao et al., 1999; McGee et al., 2004; Opatowsky et al., 2003). Early studies determined that the β subunit binds with high affinity to α_1. This high affinity site is located in the cytoplasmic loop connecting the first two homologous repeats (i.e., the I-II loop) of the α_1 subunit and was named the β-interaction domain or BID (Pragnell et al., 1994) (Fig. 1.7). The BID is comprised of 18 residues with a conserved consensus motif

Figure 1.7: Structure of the β subunit.

This structure reveals the following regions: SH3 domain (Src homology 3), GK domain (guanylate kinase) and the AID (VGCCs α_1 subunit interaction domain) and the linker sequence connecting SH3 and GK domains (Vendel et al., 2006).



in all Ca_v1 and Ca_v2 α_1 subunits. The BID binds to all four β subunits (De Waard et al., 1995). The affinity of the BID-β interaction ranges from 2 to 54 nM, depending on the BID/β or α_1/β pair (Bell et al., 2001; Butcher et al., 2006; Canti et al., 2001; De Waard et al., 1995; De Waard et al., 1996; Geib et al., 2002; Opatowsky et al., 2003; Richards et al., 2004; Scott et al., 1996; Van Petegem et al., 2008). Single mutations of several conserved residues in the BID greatly weaken the BID-β interaction, as indicated by *in vitro* binding experiments and by the reduction or abolishment of β-induced stimulation of Ca^{2+} channel current in heterologous expression systems (Berrou et al., 2002; Berrou et al., 2005; Butcher et al., 2006; De Waard et al., 1996; Gerster et al., 1999; Gonzalez-Gutierrez et al., 2008; Hidalgo et al., 2006; Leroy et al., 2005; Pragnell et al., 1994; Van Petegem et al., 2008).

The core of the β subunit contains an SH3 and a GK domain, which are connected by a linker region (Chen et al., 2004; Opatowsky et al., 2004; Van Petegem et al., 2004). The β subunit structures show that β-GK binds tightly to the BID in α_1 (Chen et al., 2004; Opatowsky et al., 2004; Van Petegem et al., 2004).

The β-SH3 domain has a similar fold as SH3 domains, but its last two β sheets are noncontinuous, separated by the linker region (Chen et al., 2004; Opatowsky et al., 2004; Van Petegem et al., 2004). The β-SH3 domain contains a well preserved proline-rich motif binding site and therefore has the potential to bind proteins that contain the same motif. However in the crystal structures, this binding site is partly shielded by the linker region and a long loop connecting two of the four continuous β sheets. Thus, access to this site requires movement of these two regions. Such conformational changes are

conceivable when the β subunit is bound to the α_1 subunit. Variation among the different β subunits comes from the fact that the linker region, as well as the NH_2 and COOH termini of the β subunit are highly variable in length and amino acid composition among the β subfamilies.

In the β subunit there is a 31 amino acid segment that is referred to as the α-interaction domain (AID), which is the main binding site for the BID of the α_1 subunit (De Waard et al., 1994). The AID was able to enhance slightly Ca^{2+} current amplitude and modulate gating. Several AID point mutations were able to weaken the β/α_1 interaction and reduce AID-stimulated Ca^{2+} currents (De Waard et al., 1994; De Waard et al., 1996). However it was later found that the BID does not bind to the AID as was originally thought (Chen et al., 2004; Opatowsky et al., 2004; Van Petegem et al., 2004). Instead, the BID binds to a hydrophobic groove in the GK domain termed the AID-binding pocket (ABP) (Chen et al., 2004; Van Petegem et al., 2004, Van Petegem et al., 2008). The AID occupies only a tiny fraction of the β subunit's surface area, raising the possibility that other domains of the β subunit are involved in interactions with other regions of the α_1 subunit or with other proteins.

The BID-GK domain interactions are extensive and predominantly hydrophobic. These interactions account for the affinity measured in the BID-β binding. Functional studies show that mutating two or more key residues in the ABP severely weakens or completely abolishes the BID-β interaction (He et al., 2007; Zhang et al., 2008).

The binding of the BID with β does not significantly alter the β subunit's structure, except for some small and localized changes near the ABP. Importantly, however, the BID undergoes a dramatic change in secondary structure when it is engulfed by the ABP. When alone, the BID exists as a random coil in solution, as determined by circular dichroism spectrum measurements (Opatowsky et al., 2004). When bound to the β subunit, the BID forms a continuous α-helix. Together with the observation that the 22-amino acid linker between the BID and the first S6 segment of α_1 (i.e. IS6) also forms an α-helix (Arias et al., 2005), a picture emerges that the entire region encompassing IS6 and the BID adopts a continuous α-helical structure in the presence of the β subunit. This structural hallmark is crucial for the regulation of calcium channel gating by the β subunit.

D. The Lethargic *(lh)* Mutation

The *lethargic* locus encodes the VGCCs β_4 subunit, and is the first example of a mammalian neurological disease caused by an inherited defect in a non-pore forming subunit of a VGCCs (Burgess et al., 1997). The *lh* mutation is caused by a four-base pair insertion into a splice donor site within the *Cacnb4* gene on mouse chromosome 2 that produces two abnormally spliced mutant mRNA isoforms. Both of these result in translational frameshift and a premature stop codon. The predicted β_4 protein is truncated - missing 60% of the C-terminal of the β_4 protein, including the AID (Fig. 1.8) (Burgess et al., 1997). The predicted β_4 protein is likely to be completely non-functional, and the *lh* mutation can thus be considered a β_4 null mutant. A second allele, lh^{2J}, mutated in the

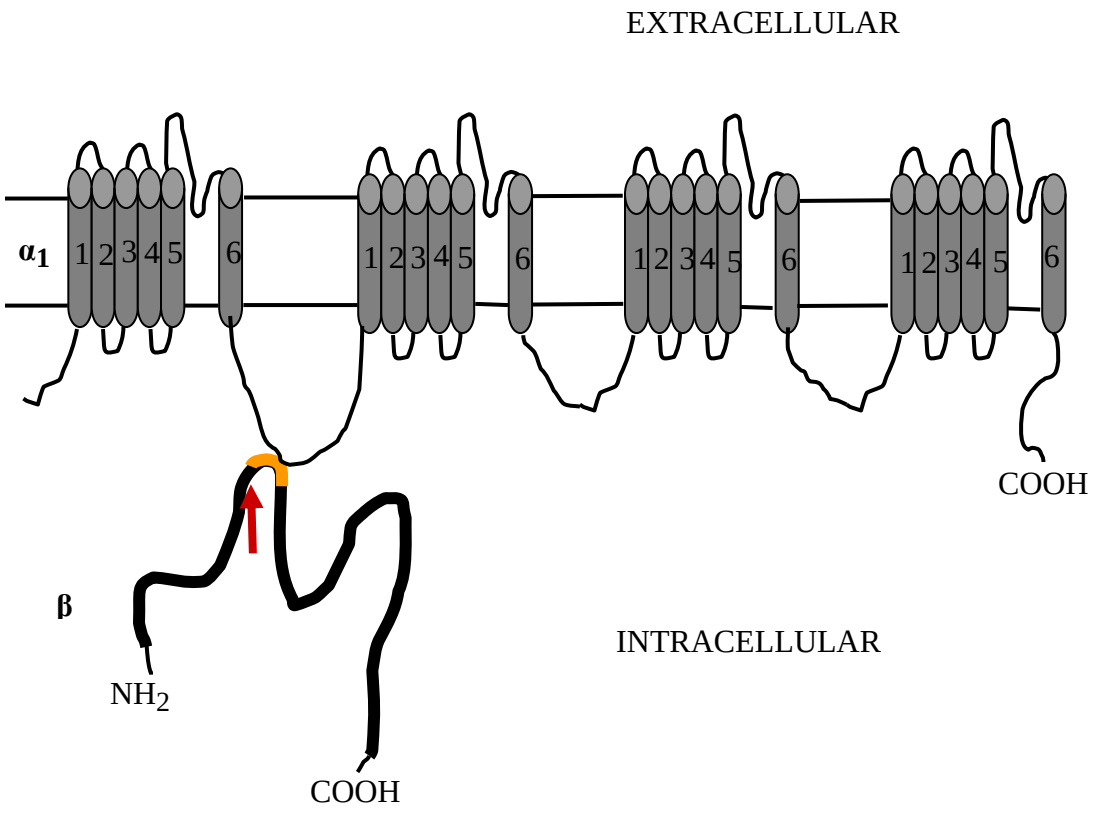
promoter region, appears to eliminate β_4 mRNA expression and results in a mutant phenotype identical to that seen in t*ottering*, including spike-wave epilepsy, ataxia, and paroxysmal dyskinesia. The onset of ataxia is 2 weeks after birth. In addition to the neurological disorders, *lethargic* homozygotes show a slightly reduced body weight and transient defects in nonneuronal tissues including the immune system, splenic and thymic involution, and renal cysts (Dung, 1977; Dung and Swigart 1971, 1972). Electrophysiologically and pharmacologically, these seizures are similar to the *absence* seizures present in the human *petit mal* epilepsy and to those present in *tg* mice. Neuropathological observations in the *lethargic* mouse brain show reduction in the size of the cerebellar molecular layer. Regional increases in the levels of $GABA_B$ receptor binding have been reported (Lin et al., 1993), providing an example of the downstream responses that may occur in a brain attempting to compensate for a persistent physiological abnormality. Additionally, T-type VGCC upregulation (by ~50%) in thalamic neurons of *lh* mice has been reported, and hypothesized that they might contribute to the seizures (Zhang et al., 2002). These mice also present lower N-type channel expression in the forebrain and cerebellum (McEnery et al., 1998), reduced excitatory neurotransmission in the thalamus (Caddick et al., 1999).

E. Regulation of Calcium Channels By Small G Proteins

RGK is a family of small GTP-binding proteins, which can regulate VGCCs' function and surface expression. RGK has four members – Rad, Gem (and its mouse homolog Kir), Rem, and Rem2, all of which have a conserved Ras GTPase-like core (Kelly, 2005). RGK proteins present the following characteristics: (1) they have low

Figure 1.8: Interaction of VGCCs α_1 subunit and β subunit.

The binding site for the α_1 I-II cytoplasmic loop is indicated by the orange segment of the β subunit. The red arrow indicates the relative position of the 4 base-pair insertion in the intron, resulting in exon skipping, translational frameshift and posterior protein truncation of the *lh* Cchb4 proteins (Adapted from Burgess et al., 1997).



intrinsic GTPase activity, secondary at least in part to modifications of key amino acids involved in GTP binding and hydrolysis in Ras GTPases (Chen et al., 2005; Kelly, 2005), (2) they have an extended N-terminal with a 14-3-3 binding domain and an extended C-terminal domain that contains binding sites for 14-3-3 and Ca^{2+}/calmodulin (Béguin et al., 2005; Béguin et al., 2006; Kelly, 2005). All members of the RGK family have the ability to interact with the β subunit. Rem2, however, has a unique characteristic in that it is the only member of the RGK family expressed in high levels in the CNS (Finlin et al., 2006). Another member of this family, Gem, was shown to inhibit potently the trafficking of $Ca_V1.2$ channels to the cell surface in HEK293 cells (Béguin et al., 2001), a finding subsequently demonstrated for the regulation of calcium channels by Rem2 (Béguin et al., 2005) as well as Rad and Rem (Béguin et al., 2006). It was proposed that the subcellular distribution of all the members of the RGK family is regulated by 14-3-3 proteins and calmodulin (Béguin et al., 2001; Béguin et al., 2005; Béguin et al., 2006). Hence, the abilities of RGK proteins to sequester the β subunit within the ER are under dynamic control, thus allowing 14-3-3 proteins and calmodulin to regulate indirectly the trafficking of the α_1 subunits, and thus their membrane expression.

In addition to their role in trafficking, it has been shown that RGKs have the ability to potently inhibit high-voltage Ca^{2+} currents. Exogenous overexpression of RGK proteins in heterologous expression systems and neurons consistently demonstrates almost complete inhibition of VGCC currents (Béguin et al., 2005; Chen et al., 2005; Finlin et al., 2003), with further experiments in *Xenopus* oocytes demonstrating that this may be a concentration-dependent effect (Seu and Pitt, 2006). It was recently shown that

the VGCC subunit β_{2a}, within its highly conserved GK domain, contains separate sites for interacting with the α_1 subunit (via the AID domain) and Rem (and even maybe with other members of the RGK family) (Finlin et al., 2006). Furthermore, the β subunit can simultaneously bind both the BID motif of the α_1 subunit and Rem. Moreover, it was recently shown that a β subunit is required for Gem and Rem2 inhibition of $Ca_V1.2$ (Seu and Pitt, 2006). This suggests that the RGK regulation of VGCCs function is not due to a physical uncoupling between the α_1 and β subunits, but may instead be due to an alteration of the functional properties of α_1/β subunit complexes. In support of such a mechanism, electrophysiological studies (Chen et al., 2005) suggest that Rem2 interaction with the N-type calcium channel results in a non-conducting β subunit containing complex. Taken together, these data suggest that in some cases, RGKs have two roles in regulating VGCCs activity: they alter membrane trafficking (independently, and via calmodulin and 14-3-3), and they directly inhibit membrane-associated channel complexes.

G. Specific aims

In *wt* mice ACh release is primarily controlled by P/Q-type VGCCs in which the α_{1A} subunit normally coassociates with the β_4 subunit. The interaction of the correct β subunit with its corresponding α_1 subunit is essential for proper targeting, membrane insertion, channel density, channel kinetics and interaction with vesicular release proteins (Murakami et al., 2003; Wittemann et al., 2000).

lh mice have a naturally occurring mutation in which there is a four base pair insertion into a splice donor site within the Cacnb4 gene on mouse chromosome 2. This mutation results in exon skipping, translational frameshift, and protein truncation missing 60% of the C-terminal relative to *wt*, including the loss of the AID site, suggesting that this defect in the VGCC assembly might be the cause of the pathogenesis in the *lh* phenotype (Burgess et al., 1997; Burgess and Noebels, 1999). This phenotype is characterized by ataxia, lethargic behavior, spike-wave epilepsy, and paraxysomal dyskinesia. The onset of ataxia is two weeks after birth. Additionally, *lh* mice show reduced body weight and immunological problems when compared with unaffected litter mates (Sidman et al., 1965); only 20% of homozygous *lh* mice survive after the weaning period.

β_4 mainly coassociates with α_{1A} subunit of the neuronal P/Q-type VGCC (McEnery et al., 1998), therefore it is not a surprise that *lh* mice have a similar phenotype to *tg* mice which carry a mutation in the α_{1A} subunit. Hence, it is normal to speculate that the β_4 mutation present in *lh* mice is having an effect on α_{1A} subunit of the neuronal P/Q-type VGCC. The overall goal of this dissertation is to characterize the *lh* mutation. How does the absence of the β_4 subunit affect the ACh release, and which compensatory mechanisms (if any) are present in *lh* mice to compensate for this mutation in these animals? To answer these questions, I propose to examine the following specific aims:

Specific Aim 1:

VGCCs are involved, among other functions, in controlling ACh release from motor nerve terminals. The *lh* mutation disrupts the normal conformation of the neuronal P/Q-type VGCC, in which the β_4 subunit is absent in animals with this mutation. Chapter 2 deals with determining how ACh release at motor nerve terminals is affected by the alteration of the normal complement of calcium channel β subunit. For this purpose intracellular recordings at the neuromuscular junction of adult *lh* and *wt* mice were obtained. Sr^{2+} and Ba^{2+} can substitute for Ca^{2+} in asynchronous spontaneous release since the Ca^{2+} selectivity filter of α_1 of VGCCs allows for the binding of these 3 divalent cations. Moreover, the Ca^{2+} permeability pore is more permeable to Sr^{2+} and Ba^{2+}, and Sr^{2+} and Ba^{2+} have a higher frequency of spontaneous release as compared to Ca^{2+}. Therefore if this mutation was affecting in a subtle way certain characteristic of the channel, using cations that are more sensitive than Ca^{2+} would allow me to determine possible changes in the channel's characteristics. Therefore, I measured spontaneous release (MEPPs) of ACh in the presence of different divalent cations (Ca^{2+}, Sr^{2+} or Ba^{2+}) and evaluated whether this mutation alters in any way the frequency or amplitude of spontaneous release. I also measured nerve evoked release (EPP) of ACh (with the buffer containing either Ca^{2+} or Sr^{2+} as the charge carrier) and examined whether this mutation altered the EPP amplitude or the quantal content. To have a further understanding on how this mutation might affect ACh release I measured the kinetics of

the synaptic vesicle release by means of FM1-43 fluorescence. Destaining, as a measure of synaptic vesicle release, was induced by high KCl (40 mM KCl), and the destaining process was measured. Neurotransmitter release can be induced by high KCl or by nerve stimulation. A high KCl solution induces massive asynchronous exocytosis, while synchronous exocytosis can be induced by stimulation of the motor nerve. This allowed me to evaluate whether this mutation affected the synaptic vesicle kinetics. Additionally, ACh release can be induced in the absence of Ca^{2+} by either high osmolarity conditions (Palma et al., 2011; Sons and Plomp, 2006) or in the presence of α-latrotoxin (α-LTx) (Hubbard et al., 1968; Sons and Plomp, 2006; Rosenmund and Stevens, 1996; Rossetto et al., 2004; Tedesco et al., 2009; Xu et al., 2002). These sets of experiments allowed me to evaluate the kinetics of release in the absence of Ca^{2+}.

Specific Aim 2:

Chapter 3 determined whether the P/Q-type calcium channel is expressed at motor nerve terminals of *lh* mice. As a first step, I tried to assay for protein level in diaphragm, however, due to the scarcity of VGCCs proteins present in this tissue and the poor sensitivity of western blots I was unable to probe against any subunit (no bands were revealed in these blots). Therefore, to answer this question I performed western blots on proteins isolated from the cerebellum of *lh* and *wt* mice and probed for the different β subunits as well as α_{1A}. Since β_4 is normally coassociated with α_{1A}, then it is plausible that a mutation affecting β_4 might alter the levels of α_{1A}. Additionally this allowed me to evaluate whether the protein level of any other β subunit was altered in response to this mutation. I also performed intracellular recordings in *wt* and *lh* adult mice in the presence

of ω-agatoxin IVA, a specific antagonist of the P/Q-type calcium channel, to determine whether the P/Q-type calcium channel controls ACh release in *lh* mice, and if so, whether its involvement in ACh release is to the same extent as it is in *wt* mice. All these results were corroborated with immunohistochemistry assays to determine the localization of α_{1A} and the possible determination of which β subunit(s) is present at the NMJ when α_{1A} is present.

Specific Aim 3:

Chapter 3 deals with determining if there are other types of VGCCs contributing to the control of the release of ACh at motor nerve terminals of adult *lh* mice. To answer this question I performed intracellular recordings in *wt* and *lh* adult mice in the presence of specific antagonist for the different types of calcium channels (ω-conotoxin GVIA which is a specific antagonists of N-type, nimodipine to antagonize L-type and SNX-482 to block R-type calcium channels). This allowed me to evaluate the extent of the involvement of the different calcium channels in the control of ACh release. Moreover, I performed immunohistochemistry assays to determine the localization of the different α_1 subunits and the possible determination of which β subunit is present at the NMJ.

CHAPTER 2

CHARACTERIZATION OF NEUROMUSCULAR TRANSMISSION IN *LETHARGIC* (*lh*) MICE

Abstract:

Voltage gated calcium channels (VGCCs) are heteromultimeric complexes composed of α_1, β, and $\alpha_2\delta$ subunits. The β subunits have a role in directing the VGCCs complexes to the plasma membrane as well as regulating its channel properties. The α_1 subunits make up the selective pore for Ca^{2+} and determine most of the subtype-specific attributes of VGCCs. A mutation in the β_4 subunit of the P/Q-type ($Ca_v2.1$) calcium channel present in *lethargic* (*lh*) homozygous mice causes ataxia and lethargic behavior at 15 days of age. To determine how acetylcholine (ACh) released at motor nerve terminals is affected by this mutation, electrophysiological recordings at neuromuscular junctions (NMJs) were compared from adult *lh* mice with *wild type* (*wt*). Different physiological saline solutions were used for the recordings and the quantal content of the *lh* mice was compared to that of controls using $CaCl_2$ (2, 4, 8 mM), and $SrCl_2$ (2, 4 mM). *lh* responses were reduced by: 72%, 59%, 32%, 41% and 50%, respectively. The spontaneous release of ACh, measured as the frequency of occurrence of miniature end plate potentials (MEPPs) remained unchanged for the various Ca^{2+} concentrations, but a 54% and 52% reduction was observed when using 2 mM and 4 mM $SrCl_2$, respectively. A 56% and 57% reduction in the spontaneous release of ACh was observed when using $BaCl_2$ (0.5, 1 mM) respectively, in the *lh* mice as compared to control animals. The reduction in the ACh level could be due to a slower process of release of the neurotransmitter vesicles. To determine the vesicle dynamic process, we used the FM1-

43 staining method. We determined that the vesicle dynamic process is severely affected by the *lh* mutation. 3.5 minutes of external stimulation by high KCl (40 mM) in *wt* mice caused an almost complete destain of the FM1-43 fluorescence. In *lh* mice under the same conditions after the same amount of time 38% of stain remained. This shows that the vesicle release process is significantly slower in *lh* mice as compared to *wt*. Inasmuch as the β_4 subunit is normally associated with the α_{1A} at mammalian NMJs, its disruption in *lh* mice might be anticipated to result in aberrant neuromuscular transmission.

Introduction:

Voltage gated calcium channels (VGCCs) are formed by α_1, β, and $\alpha_2\delta$ subunits, and some times γ (Tsien et al., 1991), where the α_1 subunits make up the selective pore for Ca^{2+} and determine most of the subtype-specific attributes of the channel. The α_1 subunit contains binding sites for various pharmacological agents as well as the gating regions of the channel (Catterall et al., 2005; Zhang et al., 1993). The cytoplasmic auxiliary β subunit regulates the assembly and membrane localization of the α_1 subunits, enhances Ca^{2+} currents and modifies the voltage-dependence and kinetics of activation and inactivation (Lacerda et al., 1991; Stea et al., 1993). It also has antagonistic effects on Ca^{2+} currents by regulating different aspects of channel function. The β-subunit contains a guanylate kinase (β-GK) and a Src homology 3 (β-SH3) domains. While β-GK binds to a conserved site within the α_1-pore-forming subunit and facilitates channel opening, β-SH3 also promotes endocytosis by binding to dynamin, a GTPase responsible for endocytosis. Channel activation and internalization are two mutually exclusive functions of the β subunit (Miranda-Laferte et al., 2011).

There are four different types of β subunit (β_{1-4}), in which the β_4 subunit normally coassociates with the α_{1A} subunit of the P/Q-type VGCC. However, there is a naturally occurring mutation of the β_4 subunit in the P/Q-type ($Ca_v2.1$) VGCCs present in *lethargic* (*lh*) mice, which causes a series of neurological signs that mimic certain

human neurological diseases. Mice homozygous for *lh* mutation exhibit ataxia, wobbly gait, smaller body size, and lethargic behavior beginning at 15 days of age (Burgess et al., 1997; Burgess and Noebels, 1999).

In neurons, the primary role of VGCCs is to mediate transmitter release from nerve terminals both in the central and peripheral nervous system by conducting presynaptic Ca^{2+} influx required for neurotransmitter release (Mintz et al., 1995; Uchitel et al., 1992). Ca^{2+} is vital for many processes, such as acting as second messengers that play crucial roles in cellular metabolism, excitability, contraction, gene regulation, vesicle cycle, hormone and neurotransmitter release (Augustine et al., 1987; Catterall, 1995; Katz and Miledi, 1970; Llinas et al., 1976; Miller, 1987).

The synaptic vesicle cycle allows these vesicles to be used repeatedly in the processes releasing neurotransmitters from chemical synapses at nerve endings. The cycle includes several stages: exocytosis (fusion of vesicles with the presynaptic membrane and secretion of a quantum of neurotransmitter into the synaptic cleft), endocytosis (formation of a new vesicle), and intracellular transport of synaptic vesicles (Zefirov, 2007; Zefirov et al., 2006). The rate of the vesicle cycle provides for efficient secretion of neurotransmitter during prolonged high-frequency synapse activity.

Ca^{2+} plays an important role in the mechanisms of the vesicle cycle. In natural conditions, the influx of Ca^{2+} through VGCCs in the active zones of motor nerve terminals triggers exocytosis (Chapman, 2008; Südhof, 2004). Ca^{2+} has both inducing and inhibiting roles in the processes of vesicle recycling (Zefirov, 2007).

The process of vesicle fusion involves different Ca^{2+}-binding sites, whose activation triggers different types of neurotransmitter secretion: synchronous, phasic (lasting a few milliseconds after the presynaptic action potential), and spontaneous asynchronous (Van Der Kloot and Molgó, 1994), which facilitates neurotransmitter secretion in conditions of high-frequency activity (Zefirov and Mukhamed'yarov, 2004). These sites differ in terms of their locations, affinities for Ca^{2+}, and sensitivities to other divalent cations, such as Sr^{2+} and Ba^{2+} (Van Der Kloot and Molgó, 1994; Zefirov and Mukhamed'yarov, 2004). The Ca^{2+} binding site for asynchronous release is sensitive to Sr^{2+} and Ba^{2+}, while the synchronous exocytosis is sensitive to Sr^{2+} only.

The β_4 subunit normally co-associates with the α_{1A} subunit of the neuronal P/Q-type VGCC (McEnery et al., 1998). Therefore, it is not a surprise that *lh* mice have a similar phenotype to *tottering* (*tg*) mice which carry a mutation in the α_{1A} subunit. The main role of P/Q-type VGCC is to mediate neurotransmitter release from nerve terminals. Hence, it is normal to speculate that the β_4 mutation present in *lh* mice is having an effect on α_{1A} subunit of the neuronal P/Q-type VGCC, which might be reflected in an alteration in ACh release from motor nerve terminals. In *lh* mice the normal complement of the α_{1A} subunit is missing, therefore, we wanted to determine how this alteration of the normal complement of the P/Q-type VGCCs affected neurotransmitter release at motor nerve terminals. To test this, we recorded miniature end plate potentials (MEPPs) and end plate potentials (EPPs) from control and *lh* NMJ to calculate the quantal content of

acetylcholine (ACh). Additionally, we tested whether this mutation affected the kinetics of release of synaptic vesicles. Vesicular release is regulated by the Ca^{2+} influx through VGCCs. Therefore, any alteration in the normal complement of VGCCs could alter the characteristics of vesicular release.

Materials and Methods:

Mice. Breeding pairs of heterozygous Cacnb4lh4J mice were obtained from Jackson Laboratory (Bar Harbor, ME) and subsequently maintained in a breeding colony at Michigan State University Laboratory Animal Resources. Litters were genotyped at weaning, 3 weeks after birth. *lh* (homozygous) mice were also identified by their characteristic phenotype consisting of a mild ataxia, wobbly gait behavior, and smaller body size. Adult mice between 3 to 9 months of age were used for all the experiments. All experiments were performed in accordance with local university (Michigan State University Laboratory Animal Resources) and national guidelines (National Institutes of Health of the USA - NIH) and were approved by the University Animal Use and Care Committee.

Electrophysiology. Animals were sacrificed by decapitation following anesthesia (80% O_2 + 20% CO_2). The diaphragm with its attached phrenic nerve was removed from each animal and pinned in a Sylgard-coated chamber. The tissue was superfused continuously with 100% oxygenated physiological saline solution (137.5 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 11 mM *d*-glucose, and 4 mM HEPES, adjusted to pH 7.4). MEPPs and EPPs were recorded from hemidiaphragms of *lh* and *wt* mice using intracellular microelectrodes (made from 1.0 mm o.d. glass capillaries; WPI, Sarasota, FL) with a resistance of 5 – 15 MΩ when filled with 3 mM KCl. Electrodes were localized at the end plate. EPPs were elicited by phrenic nerve stimulation at 0.5 Hz by means of a suction electrode attached to a stimulus isolation unit (Grass SIU5; Grass Instruments, Quincy,

MA) and stimulator (Grass S88). Signals were amplified using an Axoclamp-2 amplifier (Molecular Devices, Sunnyvale, CA), digitized using a PC-type computer and Axoscope 9.0 software (Molecular Devices, Sunnyvale, CA), and analyzed using MiniAnalysis 6.0 Software (Synaptosoft, Decatur, GA). Recordings were obtained from 5-10 end-plates for a period of 5 mins each for each mouse. This is important since there can be burst in MEPP frequency, giving rise to "clusters" of MEPPs (Fatt and Katz, 1952; Kriebel and Stolper, 1975; Vautrin and Kriebel, 1991). If the recordings are done over short periods of time these bursts of MEPPs could lead to erroneous interpretation of MEPP frequency. Sampling over a larger period of time, gives a better representation of what could naturally be occurring. Muscle action potentials were inhibited by addition of 2.5 μM of μ-conotoxin GIIIB (Alomone Labs, Jerusalem, Israel). The amplitude of MEPPs and EPPs was normalized to a membrane potential of -75 mV using the following formula: Vc = [Vo x (-75)]/E, where Vc is the corrected amplitude of MEPPs and EPPs, Vo is the actual recorded amplitude of MEPPs and EPPs and E is the membrane potential at which the recording was made (Magleby and Stevens, 1972; McLachlan and Martin, 1981). A recording was rejected if the 10-90% rise time exceeded 1.5 ms, and/or, if the membrane potential was more depolarized than -55 mV. The quantal content (*m*) was calculated using the corrected mean amplitude values of EPPs and MEPPs, where m = EPP/MEPP (Hubbard et al., 1969). We also tested ACh release in response to different divalent cations, and different concentrations of each of them. In some experiments Ca^{2+} (2, 4 or 8 mM) was substituted for Sr^{2+} (2 or 4 mM) or Ba^{2+} (0.5 or 1 mM). Separate preparations were used for each cation and concentration tested.

FM1-43 fluorescence. The thin, almost translucent *trangularis sterni* (TS) muscle preparation was chosen for these experiments because FM1-43 loads more efficiently and it can be observed more clearly. For double labeling of ACh receptors and terminals, preparations were sequentially exposed to 4 μg/ml rhodamine-conjugated α-bungarotoxin (Sigma, St. Louis, MO). The muscle was exposed to α-bungarotoxin (1:200 in physiological saline) for 20 mins, washed for 20 mins, then exposed to 8 μM FM 1-43 (Invitrogen, Carlsbad, CA) (Betz and Bewick, 1992; Betz et al., 1992; Ribchester et al., 1994) in 30 mM K^+ physiological saline solution for 3 mins, and then washed with 5 mM K^+ physiological saline for 20 – 40 mins. At least 2 terminals were examined per animal used. Destaining of the terminal was induced by perfusion of the preparation with a solution containing 40 mM KCl and 2.5 μM μ-conotoxin GIIIB. Images were obtained every 10 secs. The tissue was examined using a rhodamine filter module (excitation at 530 nm and emission of 590 nm) on a Nikon Optiphot-2 (Nikon Instruments, Tokyo, Japan) microscope equipped with a 40X water immersion objective. For observation of FM1-43, the FITC excitation filter module was used (excitation at 460 nm, emission at 520 nm). To reduce the possibility of photobleaching and phototoxicity, illumination was kept at a minimum. Images were acquired using a 40 ms exposure time. Images were captured using an Andor Ixon+ camera (Andor Technology, South Windsor, CT), connected to a PC type computer with Till Photonics software (TILL Photonics GmbH, Munich, Germany). To calculate the destaining process, the average brightness of the entire terminal was measured relative to the background. Averages of the mean values of fluorescence obtained over the different time points from all the nerve terminals sampled

were calculated and compared between the *lh* and *wt* preparations using Metamorph software (Molecular Devices, Sunnyvale, CA).

ACh release can be induced in the absence of Ca^{2+} by either hypertonic conditions (Palma et al., 2011; Sons and Plomp, 2006) or in the presence of α-latrotoxin (α-LTx) (Alomone), which induces a massive release of neurotransmitter (Hubbard et al., 1968; Rosenmund and Stevens, 1996; Rossetto et al., 2004; Sons and Plomp, 2006; Tedesco et al., 2009; Xu et al., 2002). Therefore to evaluate the kinetics of transmitter release in the absence of Ca^{2+}, we induced exocytosis by high osmolarity solution (500 mM) and in the presence of α-LTx.

α-LTX in its tetrameric form interacts with receptors (neurexins and latrophilins) on the neuronal membrane, which leads to insertion of α-LTX into the membrane. The toxin forms pores in the lipid membrane which are permeable to Ca^{2+}. This influx stimulates synaptic vesicle exocytosis. The pore is also permeable to neurotransmitters which causes massive depletion of the neurotransmitter pool in the cytoplasm (Ushkaryov et al., 2004). At nM concentrations of α-LTx, bursts of neurotransmitter release occur, followed by prolonged periods of steady-state release (Henkel and Sankaranarayanan, 1999; Ushkaryov, et al., 2008).

Hypertonic solutions induce spontaneous release of ACh from the motor nerve terminal, as reflected by an increase in MEPP frequency. The effects of osmolarity changes are not produced by changes in nerve terminal polarization (Hubbard et al., 1968). The high osmotic pressure induces dehydration of the nerve terminals, which may decrease a hydration barrier preventing synaptic vesicles from making contact with the

nerve terminal membrane and so discharging their contents (Bass and Moore, 1966).The increase in MEPP frequency maybe due to the outward flow of water through the nerve terminal (Hubbard et al., 1968)

Statistical Analysis. Differences between genotypes were analyzed using a one way analysis of variance followed by Tukey's test. Differences between concentration response were analyzed using a two way analysis of variance followed by Tukey's test. *P* values were set to < 0.05 for all tests. Measurements are expressed as means ± S.E.M for $n \geq 7$.

Results:

Spontaneous ACh release

To test whether the *lh* mutation affected spontaneous (asynchronous) release of ACh, we performed intracellular recordings from hemidiaphragms of *wt* and *lh* mice in saline solution containing Ca^{2+} (2, 4 or 8 mM) in. A change in the Ca^{2+} concentration when performing spontaneous release of ACh, translates in an increase on the frequency of occurrence. Additionally an increase in Ca^{2+} concentration, leads to an increase in vesicle release, which in turn increases recycling. Therefore if this mutation affected the frequency of release, it would be easier to visualize using a higher extracellular Ca^{2+} concentration, which would induce a faster frequency of release. We measured the amplitude and frequency of MEPPs (Fig. 2.1a and b). There was no difference in the amplitude or frequency of the spontaneous release of ACh between *lh* and *wt* mice using Ca^{2+} as a charge carrier.

Sr^{2+} and Ba^{2+} can substitute for Ca^{2+} in the generation of MEPPs (Anwyl et al., 1982; Elmqvist and Feldman, 1965; Mellow et al, 1982; Silinsky, 1978). Spontaneous exocytosis involves the activation of a low-threshold exocytosis with a calcium microdomain (Zefirov and Grigor'ev, 2008). This site is not selective and can be activated by Ca^{2+}, $Sr^{2+}$and Ba^{2+} (Zefirov and Grigor'ev, 2010). The most likely molecular candidate for this site is synaptagmin III (Chapman, 2008; Sudhof, 2004) which is a high-affinity isoform which has been shown to be able to bind to these divalent

cations (Fukuda et al., 1997; Sudhof, 2004). Using different divalent cations we could determine if characteristics of the channel were altered by this mutation, since they all have different mobility through the channel (relative conductance: $Ba^{2+} > Sr^{2+} > Ca^{2+}$) (Hagiwara and Ohmori, 1982). As such, we substituted Ca^{2+} for Ba^{2+} or Sr^{2+} in *lh* animals to determine if the release process responded differentially to these ions (Fig. 2.2a and b). The amplitude of MEPPs was not altered for either *lh* or *wt* mice by substituting Ba^{2+} or Sr^{2+} for Ca^{2+}. This implies that there is no difference in the amount of ACh each synaptic vesicle carries. However, MEPP frequency was significantly decreased in *lh* mice when used Ba^{2+} or Sr^{2+} as charge carriers.

Nerve evoked ACh release

We wanted to evaluate whether this mutation also affected the nerve-evoked release of ACh. For this purpose we performed intracellular recordings in saline solution containing Ca^{2+} (2, 4 or 8 mM) in hemidiaphragms of *wt* and *lh* mice, and measured the amplitude of EPPs evoked by nerve stimulation (Fig. 2.3). When inducing evoked release of ACh, the amplitude of the EPPs depends on the extracellular concentration of Ca^{2+}, meaning the higher the concentration of Ca^{2+} the greater the EPP amplitudes, since the change in concentration is increasing release. We observed that regardless of the concentration of Ca^{2+} we were using in the physiological saline solution, we still had a significant decrease in the amplitude of EPPs in *lh* mice as compared to *wt*. Using the amplitude of EPPs and the amplitude of MEPPs, we calculated the quantal content of the

mice (Fig. 2.4). We observed that the quantal content in *lh* mice was significantly decreased as compared to *wt*.

Strontium is capable of supporting synaptic transmission. The mechanism of phasic synchronous exocytosis includes activation of a high-threshold site mediated by the calcium microdomain close to an open VGCC (Neher, 1998; Stanley, 1993). The site for phasic exocytosis is only sensitive to Ca^{2+} and Sr^{2+}, although it has lesser affinity for Sr^{2+}, and it is insensitive to Ba^{2+} (Zefirov and Grigor'ev, 2010). This site consists of the low-affinity isoform synaptotagmin I (Sudhof, 2004). When Sr^{2+} is used for synaptic transmission replacing Ca^{2+}, peak release is reduced and the duration of release is prolonged.

When we performed the recordings in the presence of Sr^{2+} as a charge carrier, we saw that the amplitude of EPPs (Fig. 2.5) as well as the quantal content (Fig. 2.6) were also significantly decreased in *lh* mice. These results show that the *lh* mutation severely affects ACh release from adult motor nerve terminals.

FM1-43

Based on these results, we wanted to examine whether the vesicular dynamics were affected by the absence of the β_4 subunit in *lh* mice, which could explain the decrease in ACh release. These experiments are designed to investigate how the *lh* mutation affects the availability of quanta release, the exocytosis of vesicles and their recycling. Synaptic vesicle exocytosis, and internal vesicular processing were examined

using the motor nerve terminals in the *triangularis sterni* (TS) muscle of *wt* and *lh* mice. ACh depletion was achieved by massive asynchronous exocytosis induced by high KCl (40 mM KCl) solution. Then, the tissue was incubated with the fluorescent dye FM1-43, which was taken up into synaptic vesicles during the endocytic re-uptake of released vesicles. This technique can be used to estimate synaptic vesicle pool size and monitor synaptic vesicle dynamics (Betz and Bewick, 1993; Reid et al., 1999). We visualized the FM1-43 labeling dynamics observing the amount of decrease in FM1-43 fluorescence in the presynaptic terminals after KCl-induced depolarization of the nerve terminals. This translates to transmitter progressively released in real time. After 3.5 mins, most of the fluorescence was lost from *wt* NMJs, however a large amount of fluorescence remained in nerve terminals of *lh* mice. KCl evoked-released induced unloading of FM1-43 up to ~ 46.4 ± 1.6% and 8.7 ± 0.8% in *lh* and *wt* respectively (Fig. 2.7). It took ~ 15 mins for *lh* preparations to achieve a level of destaining similar to that obtained at 3.5 mins in *wt* mice (Fig. 2.8).

Additionally, to test whether this difference in the kinetics of release was due to a pool size problem or Ca^{2+} current vesicle release was induced by α-latrotoxin (α-LTx) or by hypertonic solution (500 mM). The observed reduction in ACh release may be caused by a reduction of the number of transmitter vesicles that is ready for release. In order to probe for this vesicle pool we added hypertonic medium (500 mM sucrose-Ringer) (Stevens and Tsujimoto, 1995) and measured the FM1-43 destaining process. Exposure to hypertonic solution causes exocytosis of ACh from the readily releasable pool (Stevens and Tsujimoto, 1995) and acts in a Ca^{2+} independent manner (Rosenmund and Stevens, 1996). Hypertonic solution increases the frequency (Van Der Kloot, 1991), amplitude and

duration of spontaneous release (Yu and Van Der Kloot, 1991). The rate of destaining induced by hypertonic solution was the same in *lh* and *wt* mice (Fig. 2.9). This result suggests unaltered size of the readily releasable pool at NMJs of *lh* mice.

As an alternative means to evoke transmitter release, we applied 2 μg/ml of α-LTx to *wt* and *lh* NMJs. This toxin binds to presynaptic receptors and evokes Ca^{2+}-dependent and -independent spontaneous neurotransmitter release by insertion of ion pores and stimulation of secondary messengers, emptying the readily releasable and the reserve pool together (Grishin, 1998; Rosenthal and Meldolesi, 1989; Ushkaryov et al., 2004; 2008). There was no difference between genotypes in the rate of destaining induced by α-LTx (Fig. 2.10). This result suggests that the size of both the readily releasable and the reserve pool is unaltered in *lh* mice. Therefore, destaining of FM1-43 appeared to be slower and less complete in *lh* mice. The *lh* mutation might affect the Ca^{2+} current which could result in the slow process of release.

Discussion:

Mice harboring the *lh* mutation exhibit obvious neuromuscular impairment. It has been shown that the N-terminal region of the β_{4a} isoform interacts with synaptotagmin I, and hence is involved in the vesicle release process (Vendel et al., 2006). Therefore, *lh* mice which are null for β_4 could have affected ACh release at motor nerve terminals. We studied how ACh release responded to the use of different charge carriers and different concentration of the charge carriers. In the absence of Ca^{2+}, Ba^{2+} and Sr^{2+} can support the generation of MEPPs. Sr^{2+} and Ba^{2+} act on different "sensors" in the release process (Van Der Kloot and Molgó, 1994; Zefirov and Grigor'ev, 2010; Zefirov and Mukhamed'yarov, 2004). We also wanted to understand how this mutation affected the kinetics of the neurotransmitter release, by studying the vesicle dynamics by means of the fluorescent dye FM1-43.

Results of the present study are consistent with the following conclusions: (1) ACh release following nerve stimulation (EPP) is significantly decreased in *lh* animals. (2) The quantal content is significantly reduced in *lh* mice as compared to *wt*. (3) There is a decrease in MEPP frequency in *lh* animals as compared to *wt* when using $SrCl_2$ and $BaCl_2$, but not $CaCl_2$. (4) The rate of FM1-43 destaining induced by high [KCl] is slower in *lh* mice as compared to *wt*. (5) The rate of FM1-43 destaining in *lh* animals is the same as *wt* when the release is induced either by high osmolarity or α-LTx.

Evoked ACh release from motor nerve terminals is significantly reduced in *lh* mice as compared to *wt*. The mutation had no effect on MEPP amplitude, but it does

affect MEPP frequency. Moreover, the EPP amplitude is significantly reduced in *lh* mice; leading to an overall decrease in the quantal content in these mice. These results suggest the possibility that there is a slower process of release in *lh* mice. To address this question, we used the FM1-43 staining method, which would help us in determining the vesicle dynamic process. KCl (40 mM) depolarization of *wt* mice caused almost complete destaining, reflecting a depletion of fluorescently labeled vesicles. However, under the same conditions, in *lh* mice there is still 38% of stain remaining. Thus the vesicle release process is significantly slower in *lh* mice. This result could imply that there is a problem with the vesicle pool size in *lh* mice. To determine if this was the case we induced vesicle depletion by two mechanisms: hypertonic solution and α-LTx. In both cases the rate of release was the same for both genotypes. This result implies that in *lh* mice there might be a problem in the Ca^{2+} current affecting ACh release, but that this mutation does not affect the vesicle's pool size (neither the readily releasable nor the reserve pool). It seems that the effect on Ca^{2+} current is translated in a slower vesicle dynamics which is then perceived as a decreased quantal content. Therefore in the *lh* mouse, the severe neurological phenotype might be due in part to altered vesicle dynamics.

The *lh* NMJ has not been studied extensively. Work done by Kaja et al. (2007) showed that the *lh* mutation did not affect quantal content, EPP amplitude or MEPP frequency or amplitude. Our study corroborates some of these findings, but differs in others.

The finding that *lh* mice have no difference in ACh release as their *wt* littermates proposed by Kaja (2007) differs from our results. They saw that this mutation had no effect on MEPP amplitude or frequency, or in EPP amplitude or quantal content.

However, the study of Kaja et al. (2007) did not include different divalent cations to substitute Ca^{2+} as charge carriers, which might explain why they did not see any difference in MEPP frequency, since there is no obvious difference in the frequency of spontaneous release when using Ca^{2+} as a charge carrier. However, but these differences become apparent when using Ba^{2+} or Sr^{2+}. Differences between our two studies are most likely due to age-related factors. Kaja's group use 6 wk old mice, when we carry out all our studies in mice that are between 3 – 9 mos of age. It is possible that at 6 wks of age it is too early to have full expression of VGCCs to see difference between genotypes in ACh release. Moreover, there is evidence that during the first 6 wks of life of mice there is a significant and rapid change in parameters (quantal content, resting membrane potential, MEPP frequency) (Kelly, 1978). Hence, it is important that mice are properly matched according to age. However, this would need to be examined further to substantiate the differences between our results.

The influx of extracellular Ca^{2+} through VGCCs stimulates neurotransmitter release by exocytosis. Exocytosis of synaptic vesicles occurs in the active zones enriched with VGCCs due to activation of high-affinity Ca^{2+}-site in Ca^{2+}-macrodomain (Zefirov and Grigor'ev, 2008). The β subunit regulates the assembly, membrane localization of the α_1 subunits, enhances Ca^{2+} currents and modifies the voltage-dependence and kinetics of activation and inactivation (Lacerda et al., 1991; Stea et al., 1993). *Lethargic* mice lack the β_4 subunit which is the normal complement of P/Q-type VGCCs. This alteration in the normal composition of these channels could alter the relative abundance of P/Q-type

VGCCs at motor nerve terminals, as well as Ca^{2+}currents. Both effects could explain a slower vesicle process, leading to a decrease in ACh release.

Exposure to hypertonic solution and α-LTx causes exocytosis of ACh in a Ca^{2+} -independent manner. When we induce vesicle release independently of Ca^{2+} the rate of FM1-43 destaining is similar between genotypes. This also supports the hypothesis that Ca^{2+} influx might be altered, affecting neurotransmitter release at motor nerve terminals of *lh* mice, which could account for their lethargic behavior.

Figure 2.1: Spontaneous release of ACh measured in solutions containing 2, 4 or 8 mM $CaCl_2$.

Intracellular recordings were performed in hemidiaphrams of *lh* and *wt* mice. The amplitude and frequency of MEPPs were measured for both genotypes. (a) Amplitude of MEPPs measured in hemidiaphragms of *wt* and *lh* mice. There is no difference in amplitude between genotypes. Each value represents the mean ± S.E.M of 7 animals. (b) Frequency of spontaneous release measured in *wt* and *lh* mice. There is no difference in frequency between genotypes. Each value represents the mean ± S.E.M of 7 animals.

(a)

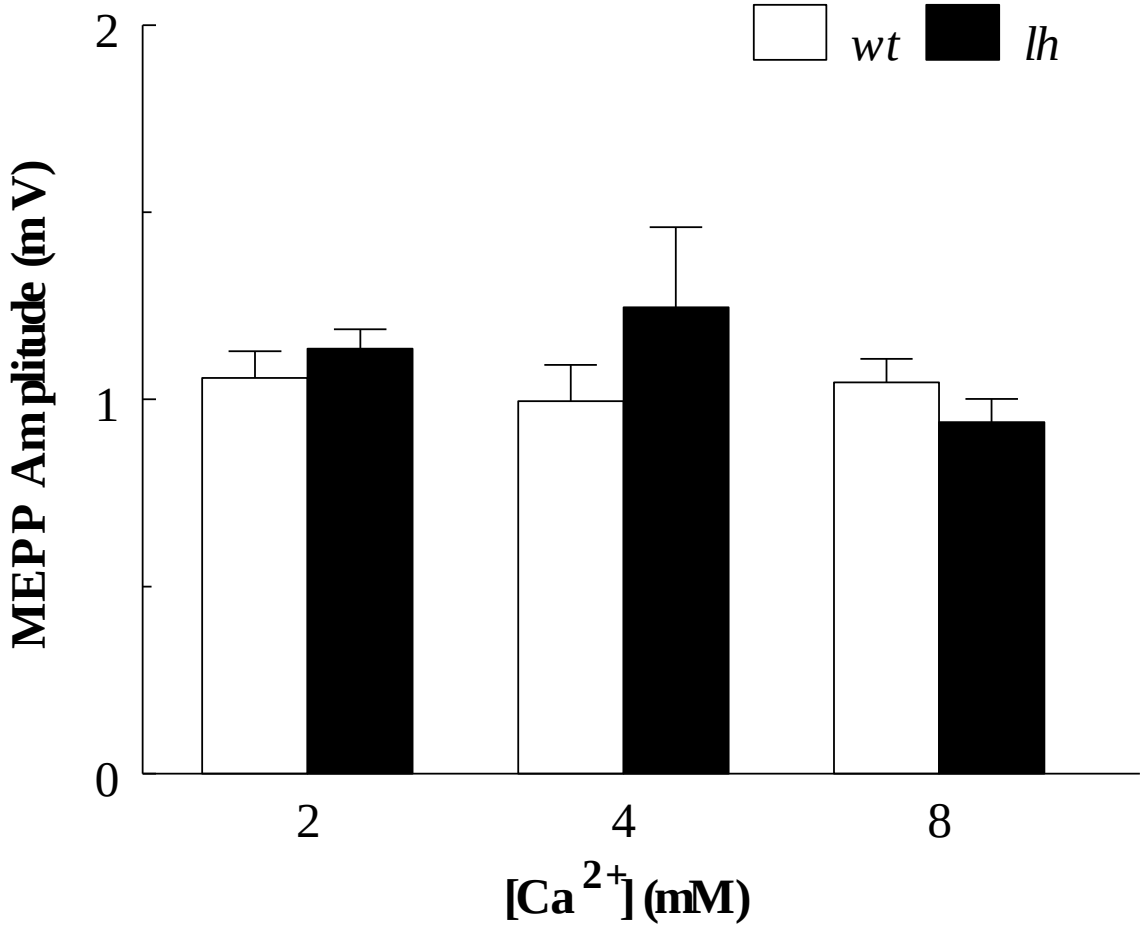


Figure 2.1 (cont'd)

(b)

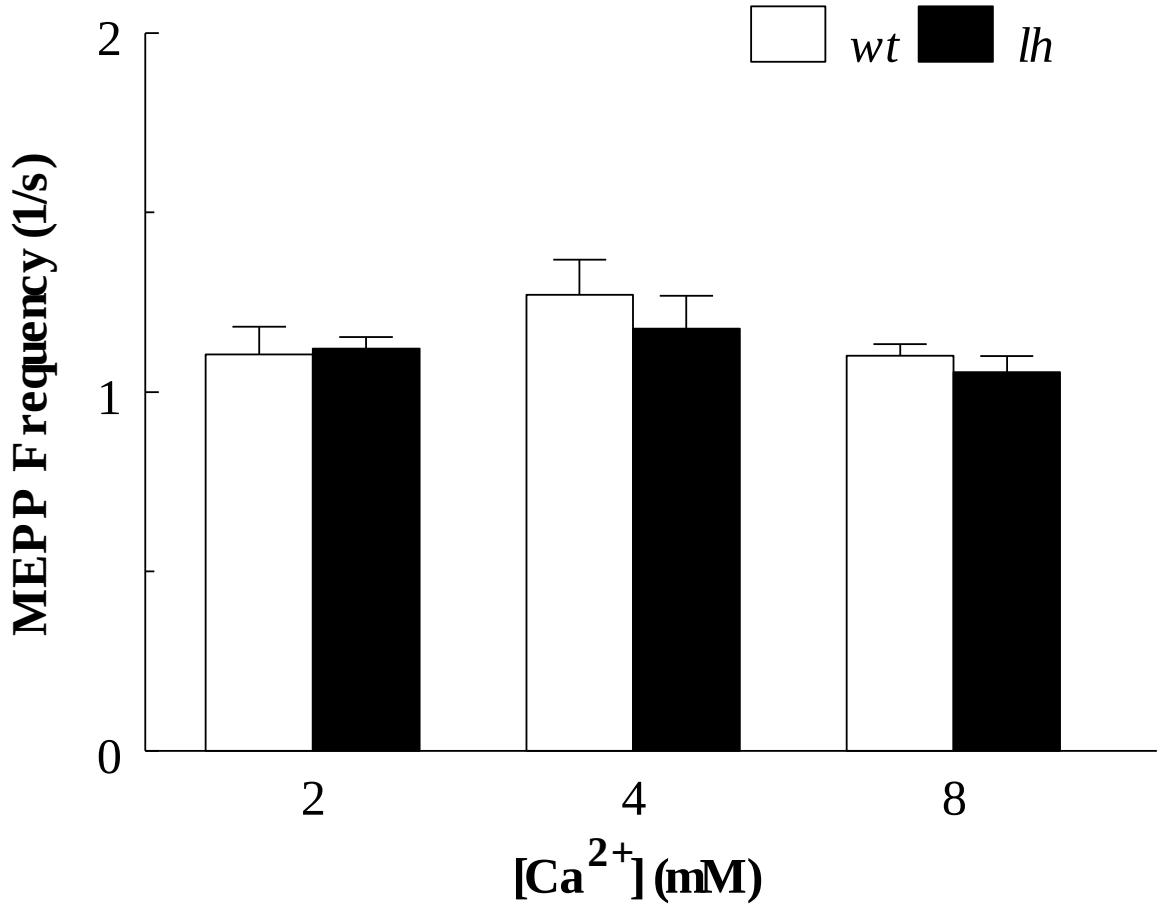


Figure 2.2: Spontaneous release of ACh measured in solution containing $SrCl_2$ (2 or 4 mM) or $BaCl_2$ (0.5 or 1 mM).

Intracellular recordings were made in hemidiaphrams of *lh* and *wt* mice. The amplitude and frequency of MEPPs were measured for both genotypes. (a) Amplitude of MEPPs measured in hemidiaphragms of *wt* and *lh* mice. There is no difference in amplitude between genotypes. Each value represents the mean ± S.E.M of 7 animals. (b) Frequency of spontaneous release measured in *wt* and *lh* mice. There is significant difference in frequency between genotypes. Each value represents the mean ± S.E.M of 7 animals. The asterisk (*) indicates a significant difference between the two genotypes.

(a)

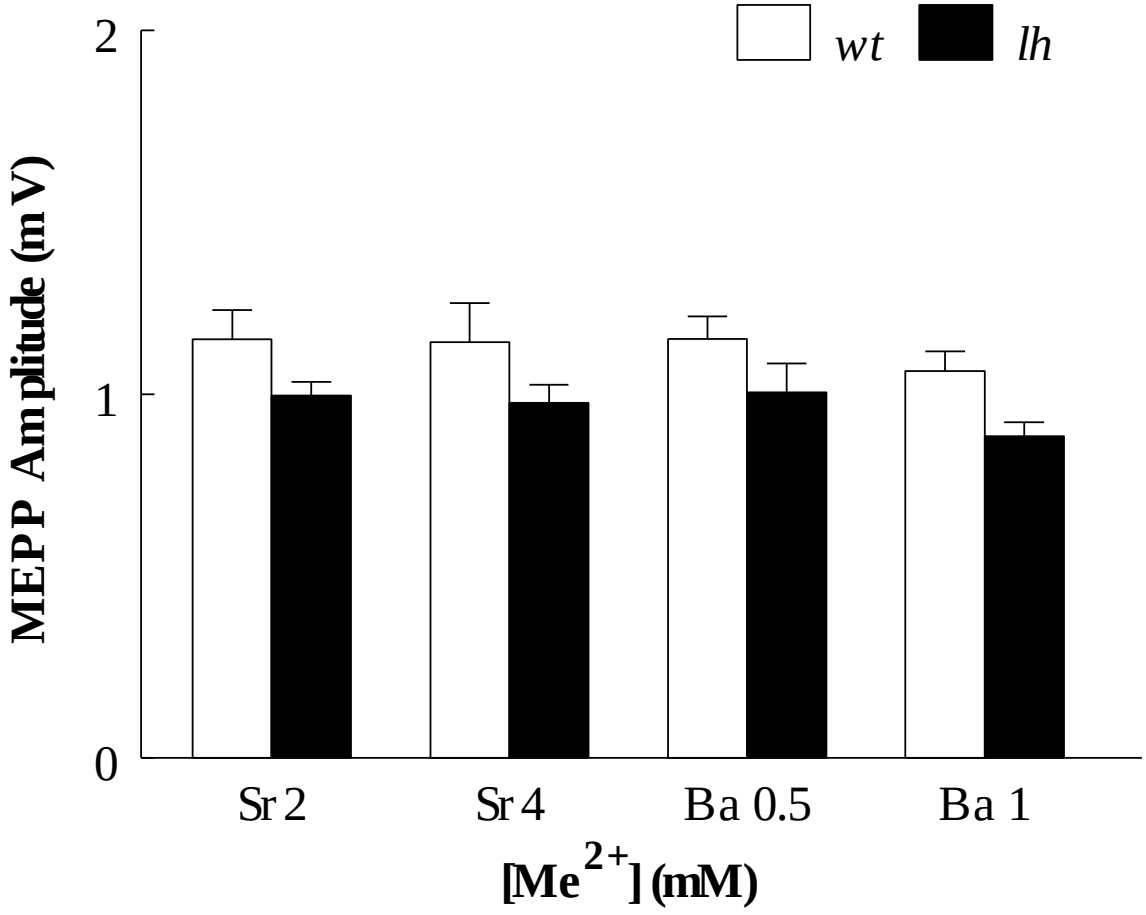


Figure 2.2 (cont'd)

(b)

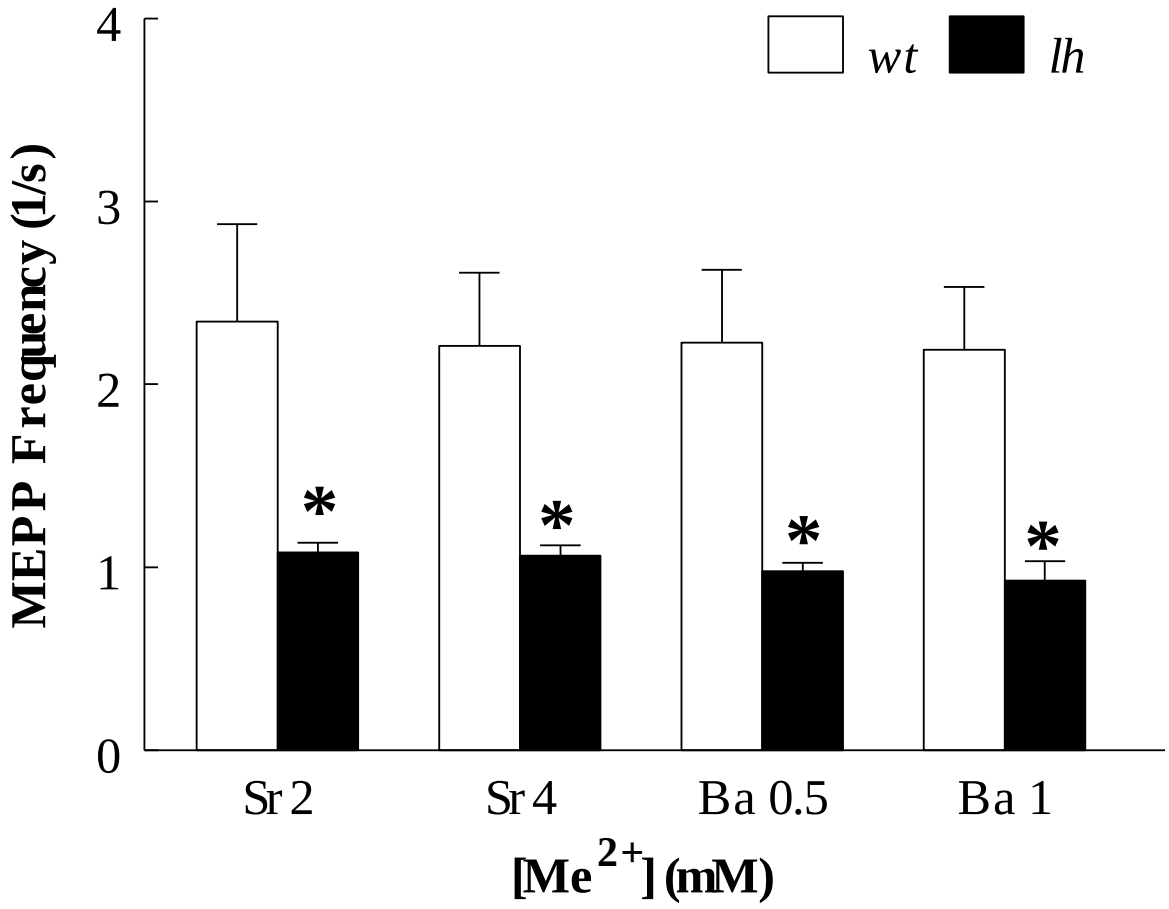


Figure 2.3: Nerve evoked release of ACh measured in solution containing 2, 4 or 8 mM $CaCl_2$.

Intracellular recordings were performed in hemidiaphrams of *lh* and *wt* mice. EPPs were elicited by phrenic nerve stimulation at 0.5 Hz by means of a suction electrode attached to a stimulus isolation unit. (a) Shows EPPs from neuromuscular junction preparations isolated from homozygote lethargic (*lh*) (orange trace) and *wt* mice (black trace). (b) The EPP amplitude was measured for both genotypes. There is a significant difference in amplitude between genotypes. Each value represents the mean ± S.E.M of 7 animals. The asterisk (*) indicates a significant difference between the two genotypes.

(a)

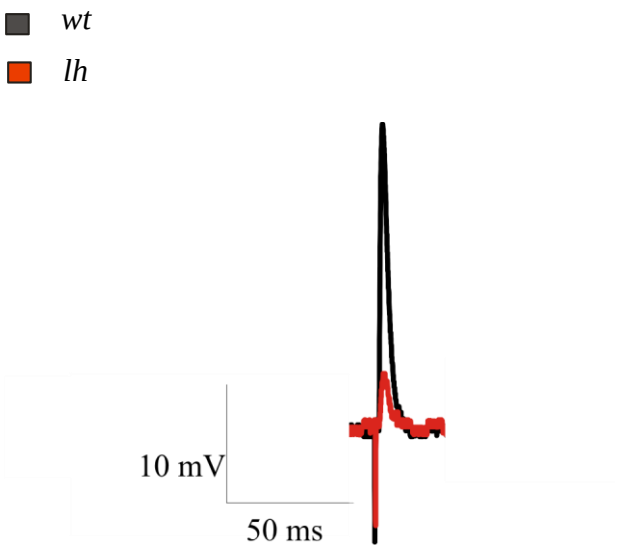


Figure 2.3 (cont'd)

(b)

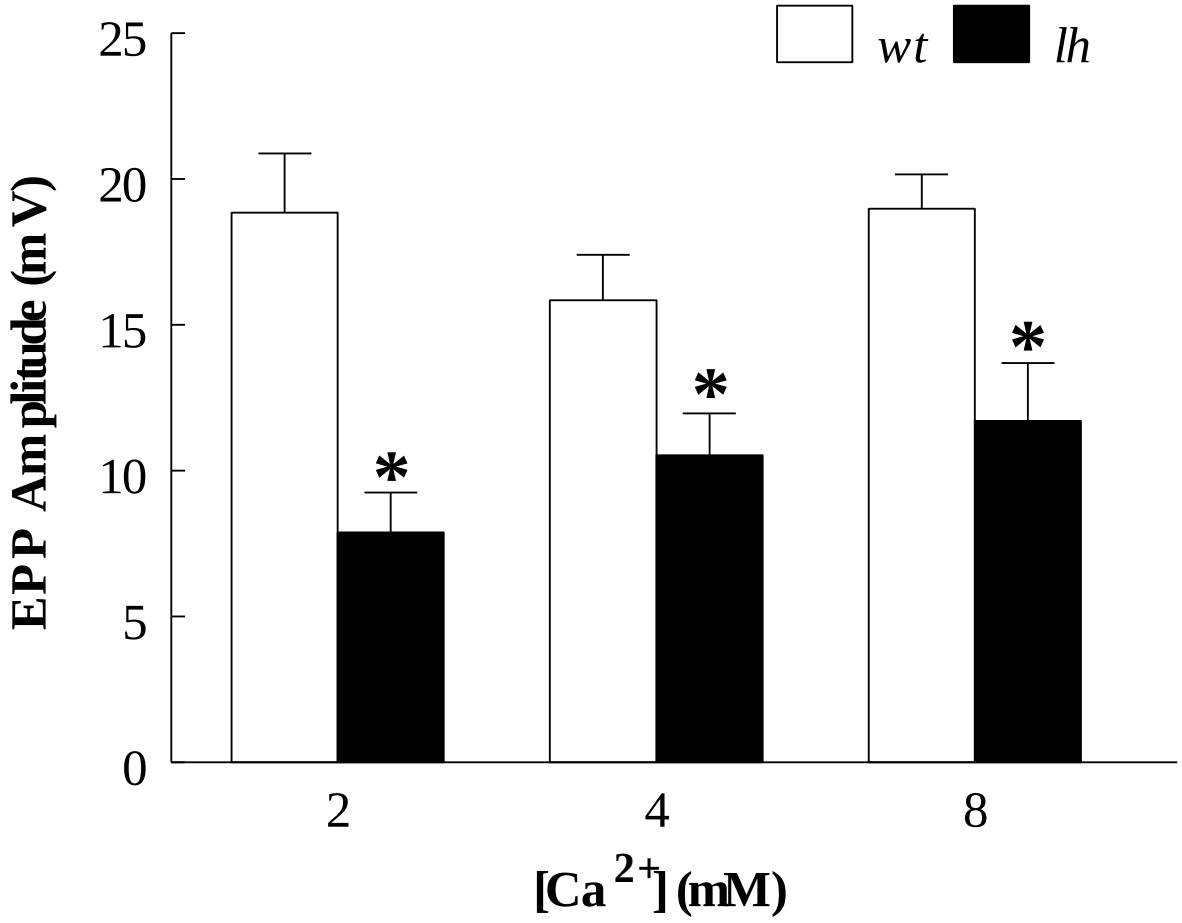


Figure 2.4: Quantal content measured in solution containing 2, 4 or 8 mM $CaCl_2$. The *m* value (quantal content) was calculated for each neuromuscular junction preparation using the ratio of the average EPPs amplitude to the average MEPPs amplitude (data from Fig. 2.1a and Fig. 2.3). The quantal content was significantly decreased in *lh* mice compared to *wt* at all Ca^{2+} concentrations used. Each value represents the mean ± S.E.M of 7 animals. The asterisk (*) indicates a significant difference between the two genotypes. The number sign (#) indicates a significant difference between Ca^{2+} concentrations.

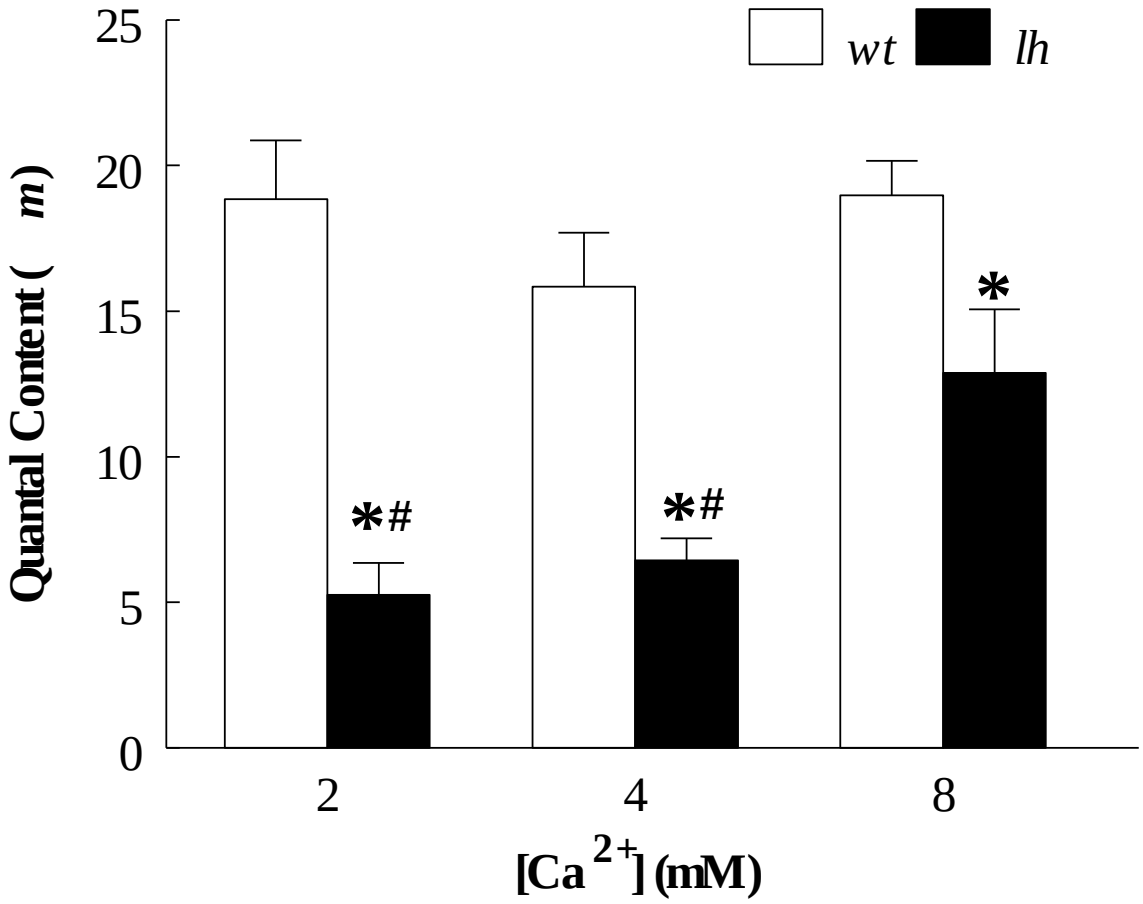


Figure 2.5: Nerve evoked release of ACh measured in solution containing 2 or 4 mM $SrCl_2$.

Intracellular recordings were performed in hemidiaphrams of *lh* and *wt* mice. EPPs were elicited by phrenic nerve stimulation at 0.5 Hz by means of a suction electrode attached to a stimulus isolation unit. The amplitude EPPs were measured for both genotypes. There is significant difference in amplitude between genotypes. Each value represents the mean ± S.E.M of 7 animals. The asterisk (*) indicates a significant difference between the two genotypes.

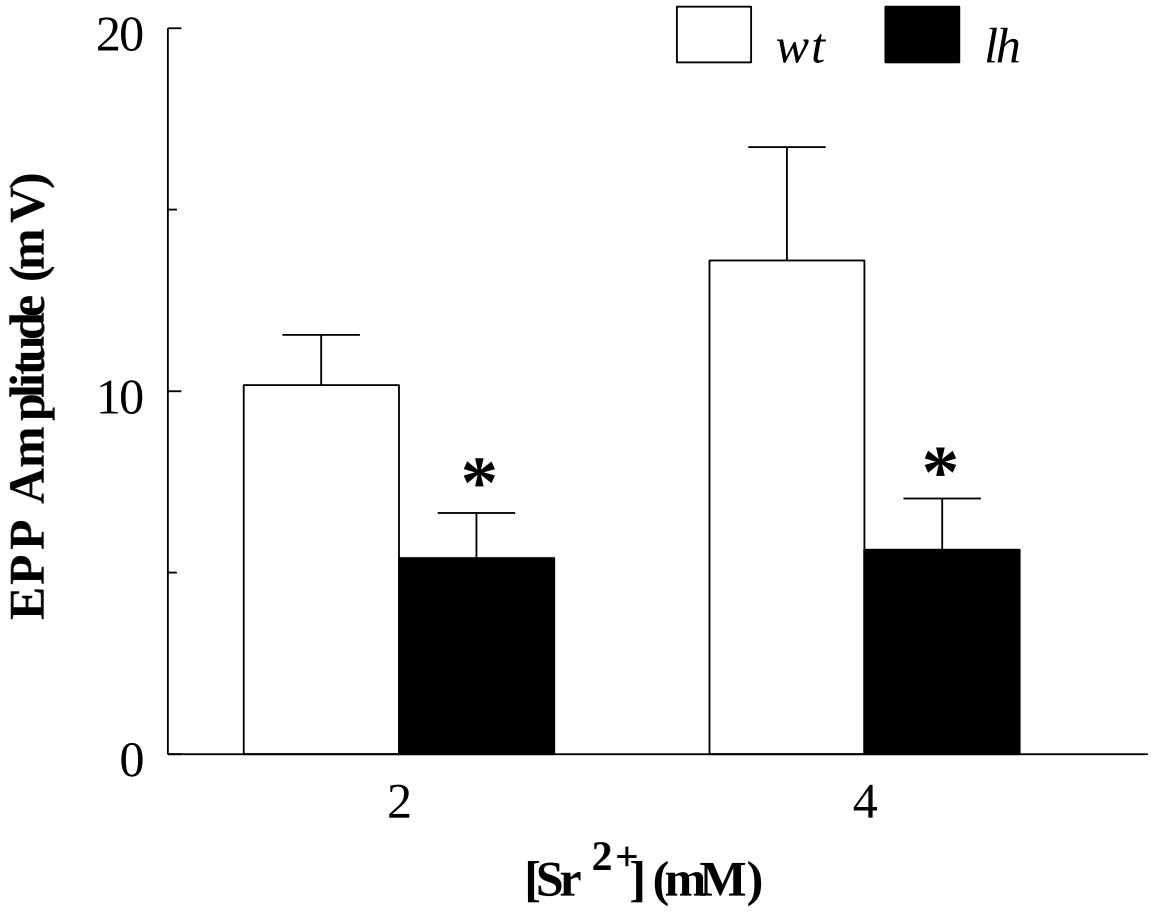


Figure 2.6: Quantal content measured in solution containing 2 or 4 mM $SrCl_2$.

The *m* value (quantal content) was calculated from each neuromuscular junction preparation using the ration of the average EPP amplitude to the average MEPP amplitude (data from Fig. 2.2a and Fig. 2.5). There is significant difference in amplitude between genotypes. Each value represents the mean ± S.E.M of 7 animals. The asterisk (*) indicates a significant difference between the two genotypes.

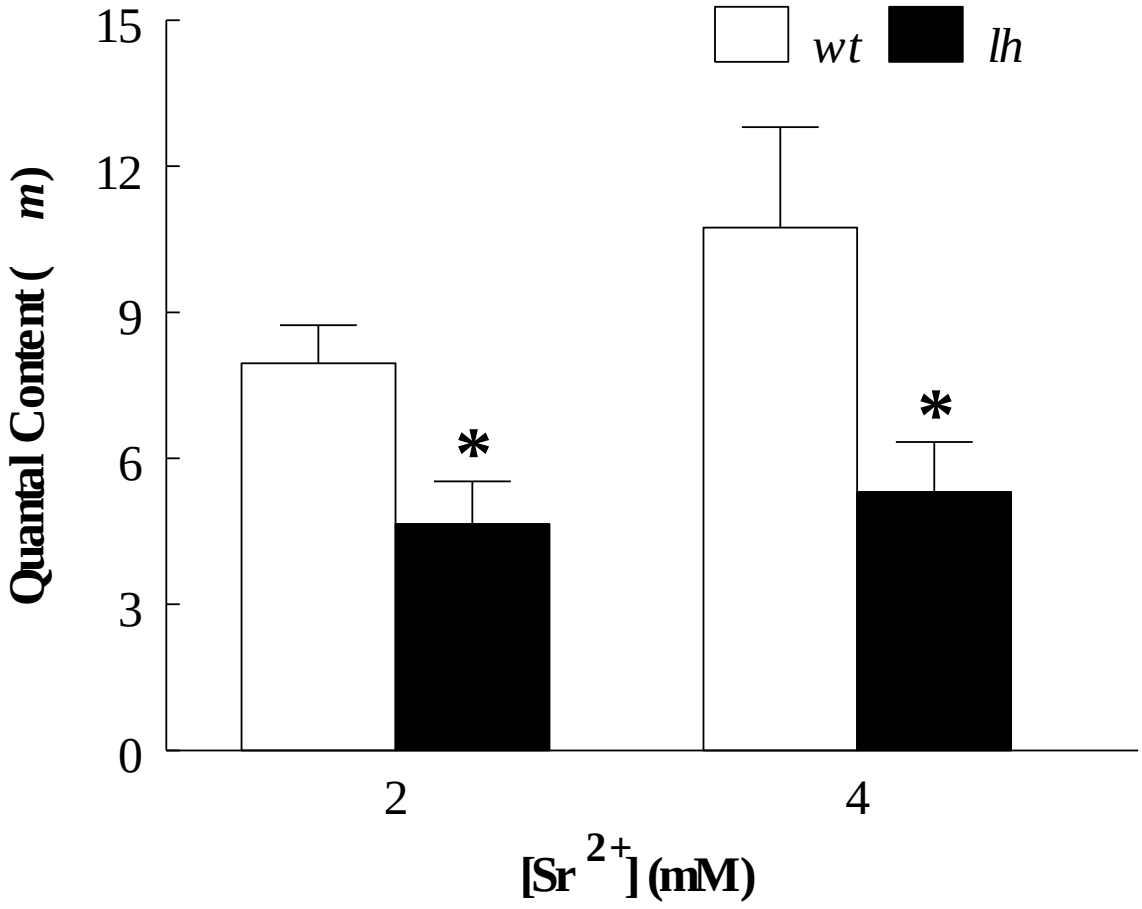


Figure 2.7: Time course of FM1-43 destaining triggered by perfusion application of 40 mM KCl in *wt* and *lh* animals.

KCl evoked FM1-43 release appears to occur faster in *wt* nerve terminals as compared to *lh*. The data are presented as the amount of fluorescence normalized to the average fluorescence values before KCl-evoked release. Each value represents the mean ± S.E.M of 9 animals. The asterisk (*) indicates a significant difference between the two genotypes.

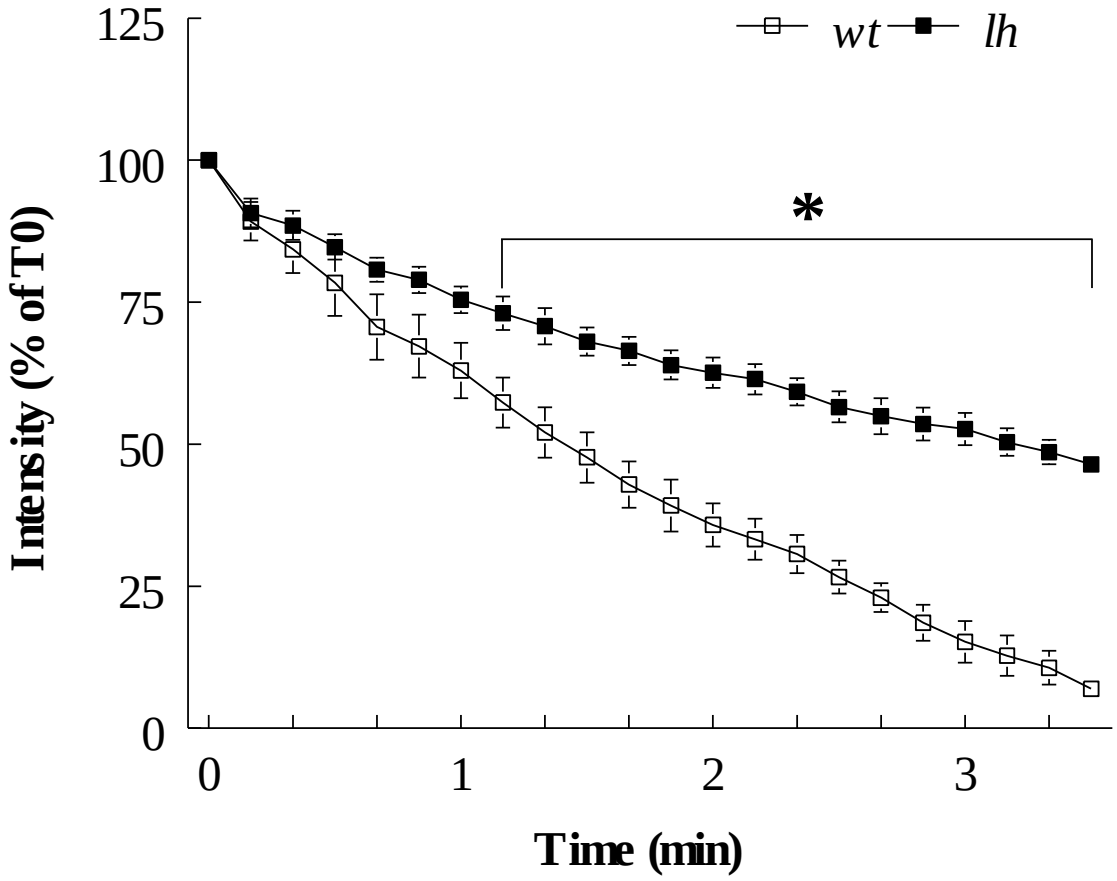


Figure 2.8: FM1-43 destaining in *wt* and *lh* motor nerve terminals.

(a) Representative images of *lh* and *wt* nerve terminals stained with FM1-43 dye, showing the distaining process evoked by 40 mM KCl over time. (b) Quantification of the destaining process in *wt* and *lh* mice. The KCl evoked FM1-43 release appears to occur faster in *wt* nerve terminals as compared to *lh*. It takes more than 15 minutes for *lh* mice to achieve a level of distaining similar to that of *wt* under the same conditions. Each value represents the mean ± S.E.M of 9 animals. The asterisk (*) indicates a significant difference between the two genotypes.

Figure 2.8 (cont'd)

(a) Representative images of a distaining process induced by 40 mM KCl, of *lh* (upper panel) and *wt* (lower panel) nerve terminal stained with FM1-43

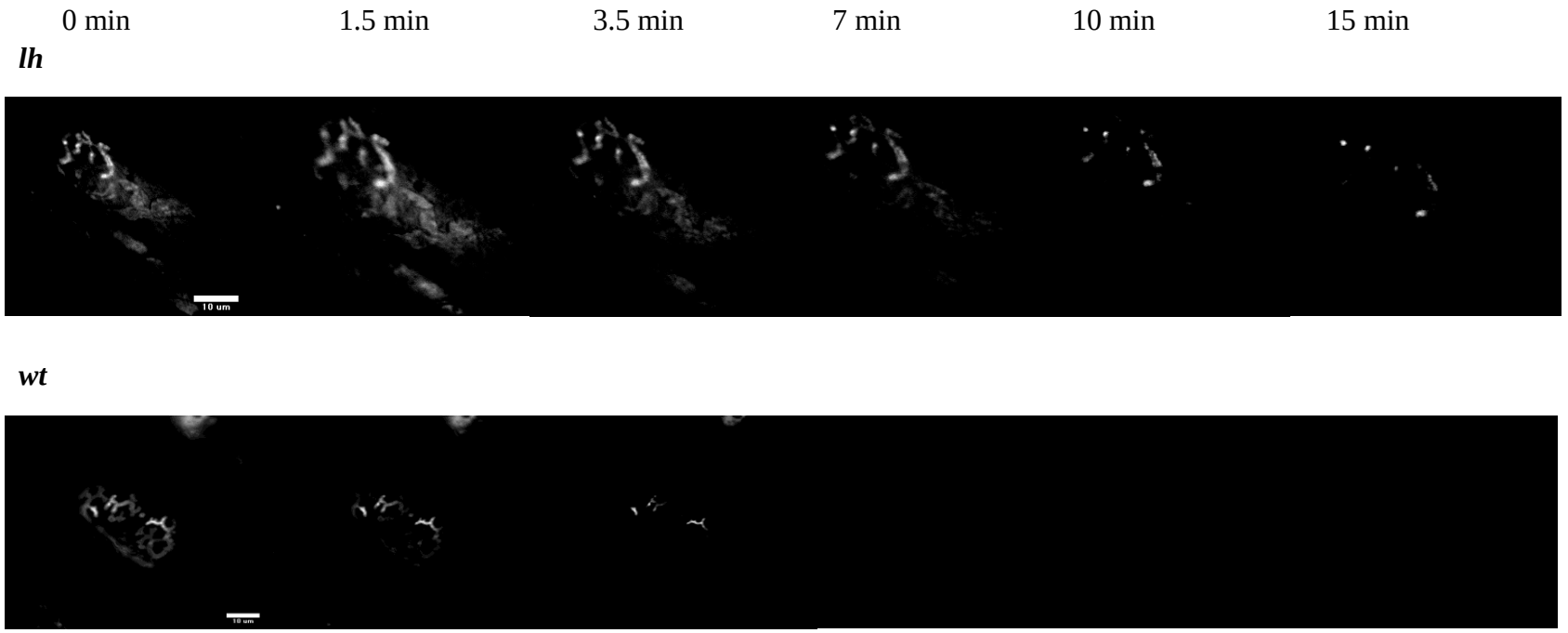


Figure 2.8 (cont'd)

(b)

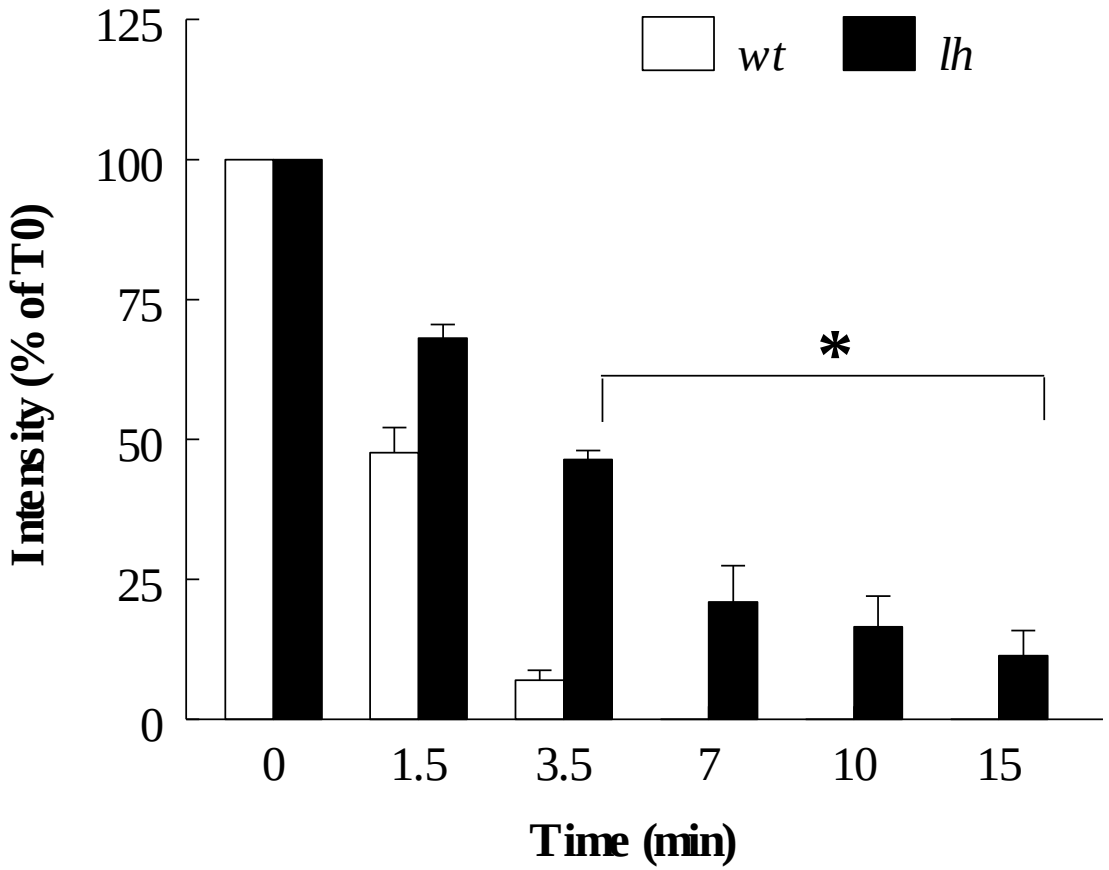


Figure 2.9: Time course of FM1-43 destaining triggered by hypertonic solution (500 mM) in *wt* and *lh* animals.

FM1-43 release evoked by high osmolarity appears to occur at the same rate in *wt* and *lh* nerve terminals. The data are presented as the amount of fluorescence normalized to the average fluorescence values before hypertonic solution-evoked release. Each value represents the mean ± S.E.M of 9 animals.

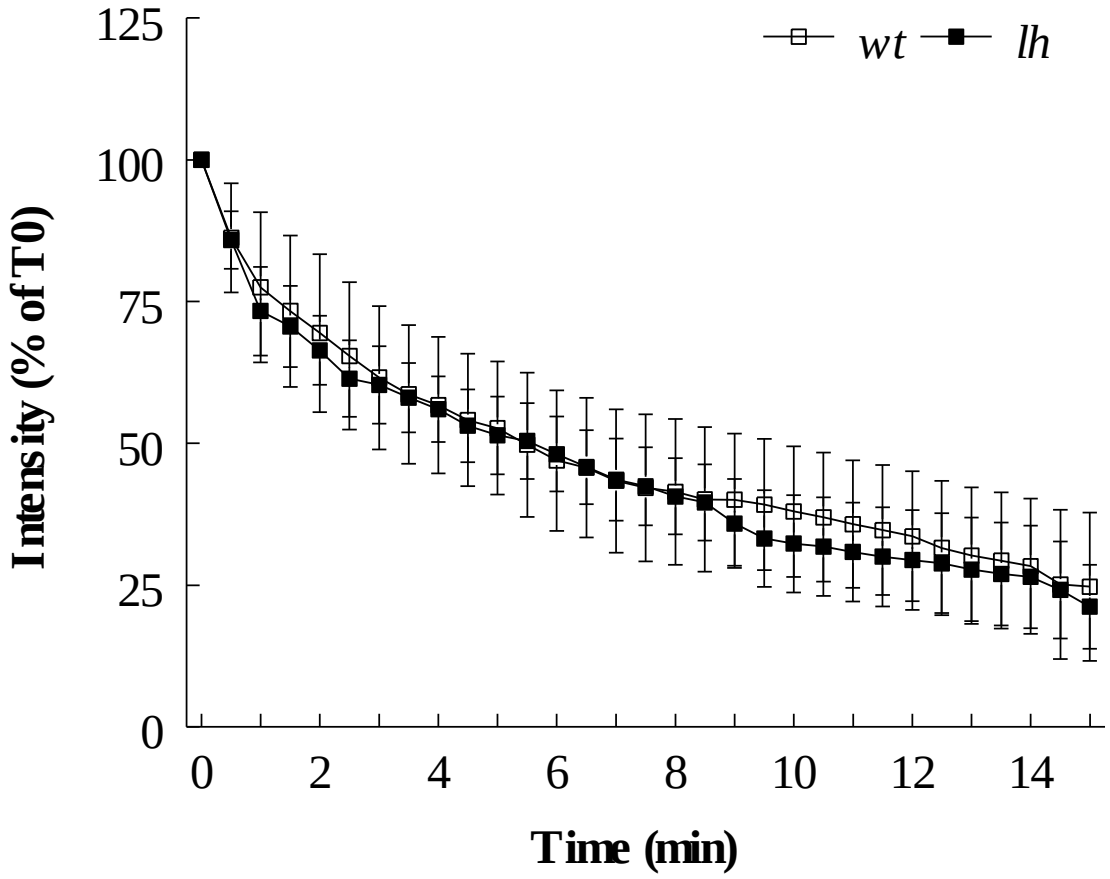
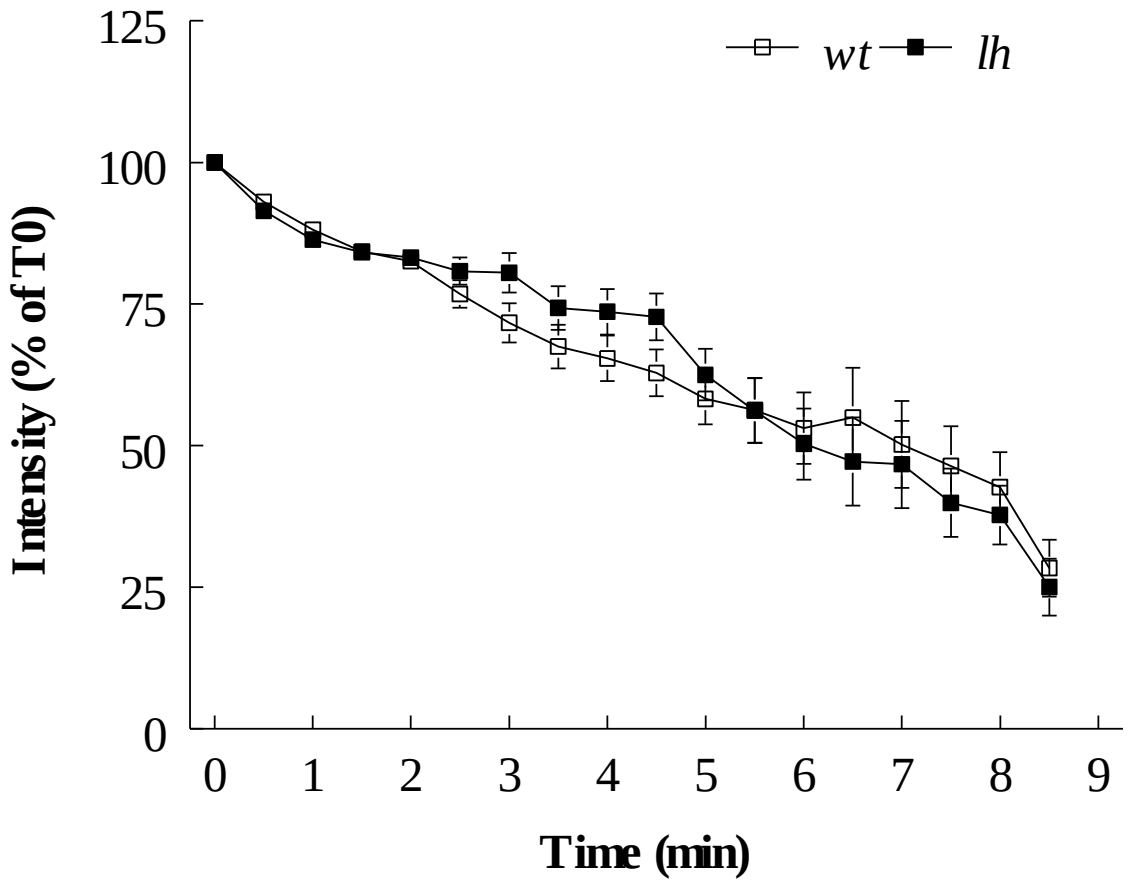


Figure 2.10: Time course of FM1-43 destaining triggered by α-LTx in *wt* and *lh* animals.

FM1-43 release evoked by α-LTx appears to occur at the same rate in *wt* and *lh* nerve terminals. The data are presented as the amount of fluorescence normalized to the average fluorescence values before α-LTx -evoked release. Each value represents the mean ± S.E.M of 9 animals.



CHAPTER 3

ACETYLCHOLINE RELEASE IS CONTROLLED BY P/Q- AND R-TYPE CALCIUM CHANNELS IN ADULT *LETHARGIC* (*lh*) MICE

Abstract:

Voltage gated calcium channels (VGCCs) are formed by α_1, β and $\alpha_2\delta$ and sometimes γ subunits. The α_1 subunit makes up the selective pore for Ca^{2+} and determines most of the subtype-specific attributes of calcium channels. This subunit contains binding sites for various pharmacological agents as well as the gating regions of the channel. The β subunit regulates the assembly and membrane localization of the α_1 subunits, and strongly influences the physiological features of the protein. The β_4 subunit normally coassociates with the α_{1A} subunit of the P/Q-type calcium channels at adult mammalian neuromuscular junctions. A natural occurring mutation present in *lethargic* (*lh*) mice due to a 4 base pair insertion into a splice donor site within the β_4 gene leads to loss of 60% of the C-terminal of the β_4 subunit relative to *wild type* (*wt*), including loss of the α_1-binding site. Mice with this mutation suffer from ataxia, lethargic behavior, spike-wave epilepsy, and paroxysomal dyskinesia. We wanted to identify which calcium channel subtypes control nerve-stimulated acetylcholine (ACh) release from motor nerve terminals of adult *lh* mice. ω-Agatoxin IVA and SNX 482 significantly reduced the quantal content in adult *lh* mice (60% and 46%, respectively), however neither toxin affected the miniature end plate potential (MEPP) frequency or amplitude. Neither ω-conotoxin GVIA nor nimodipine had any effect on ACh release. Immunolabeling of calcium channel subunits revealed an increase in α_{1E}, β_1 and β_3, but no apparent change in the levels of α_{1A} at adult *lh* neuromuscular junctions. This presumably compensates

for the absence of β_4. Therefore, it seems that in these animals the ACh release is controlled by P/Q- and R-type calcium channels, which could account for the reduction in ACh release.

Introduction:

Voltage gated calcium channels (VGCCs) contribute to entry of Ca^{2+} into neurons and muscle cells (Catterall, 1995; Katz and Miledi, 1970; Llinas et al., 1976). Although multiple VGCC types coexist in the same cell, the specific channel subtype involved in release of acetylcholine (ACh) from motor nerve terminals is both species- and age-dependent (Catterall, 1998; Rosato Siri and Uchitel, 1999). Mature mammalian motor nerve terminals contain primarily P/Q-type VGCCs (Katz et al., 1995). However, under specific conditions, subtypes of VGCCs that are not normally associated with ACh release at motor nerve terminals can mediate it (Flink and Atchison, 2002; Pardo et al., 2006; Smith et al., 1995). An example of this is seen in the *tottering* (*tg*) mutation, in which ACh release in adult *tg* mice is controlled by N- and R-type VGCCs (Pardo et al., 2006).

The different types of VGCCs can be distinguished by the genes that encode them, as well as their pharmacological and biophysical characteristics (Catterall et al., 2005; Zhang et al., 1993). VGCCs are formed by α_1, β, and $\alpha_2\delta$ subunits (Tsien et al., 1991). The α_1 subunits make up the selective pore for Ca^{2+} and determine most of the subtype-specific attributes of the VGCC. This subunit contains binding sites for various pharmacological agents as well as the gating regions of the channel (Catterall, 1995; Zhang et al., 1993). There are at least five α_1 subunits for neuronal VGCC which fall under the high voltage activated (HVA) subclass (Catterall, 1995; Tsien et al., 1991). They include: α_{1A}, α_{1B}, and α_{1E} subunits which comprise the P/Q- ($Ca_V2.1$), N- ($Ca_V2.2$), and R-type ($Ca_V2.3$) Ca^{2+} channels, respectively; while the α_{1C} or α_{1D} subunits

comprise the L-type channels ($Ca_V1.2$-1.3) (Catterall, 1995; Tsien et al., 1991). The β subunit regulates the assembly and membrane localization of the α_1 subunits. The β subunit also strongly influences the current amplitude, rate and voltage-dependence of activation and inactivation, and ligand-binding sites on the neuromuscular junction (NMJ) (Brice and Dolphin, 1999; Catterall, 1995; Walker and De Waard, 1998). There are four different types of β subunits ($\beta_{1\text{-}4}$) each encoded by a separate gene (Chien et al., 1995). The β_4 subunit is typically associated with P/Q-type VGCC (Wittemann et al., 2000). The interaction of the correct β subunit with its corresponding α_1 subunit is essential for proper targeting, membrane insertion, channel density, kinetic parameters such as activation and inactivation as well as interactions with vesicular release site proteins (Murakami et al., 2003; Wittemann et al., 2000). In the absence of the normally associating β subunit, alternate β subunits may interact with α_1 subunits to restore most of the VGCC's functions, although in an altered manner (Burgess et al., 1999).

The β_4 subunit is normally widely expressed in the brain. Spontaneous mutations in this subunit cause several neurological syndromes in mice (Burgess et al., 1997; Catterall, 1995). These mutations produce various effects in VGCCs' expression and function, such as dramatically reducing the VGCCs targeting, assembly, membrane insertion, channel density and altering characteristic kinetic parameters, vesicular release and synaptic transmission (Burgess and Noebels, 1999; Catterall, 1995; Catterall et al., 2005; Walker et al., 1998). Also, the loss of a functional β_4 subunit can impact the function of α_{1A} – containing calcium channels (P/Q-type). The *lh* mutation is caused by a

four base pair insertion into a splice donor site within the β_4 gene on mouse chromosome 2. This insertion leads to exon skipping, translational frameshift, and protein truncation, missing 60% of the C-terminal of the β_4 subunit relative to *wt*, this includes loss of the α_1-binding site, suggesting that this defect in VGCC assembly could be one cause for the pathogenesis in *lh* phenotype (Burgess et al., 1997; Burgess and Noebels, 1999). *Lethargic* mice suffer from ataxia, lethargic behavior, spike-wave epilepsy, and paroxysomal dyskinesia. Electrophysiologically and pharmacologically, the seizures are similar to the *absence* seizures present in the human *petit mal* epilepsy and to those present in *tottering* (*tg*) mice (Burgess and Noebels, 1999; Hosford et al., 1992). The onset of ataxia is two weeks after birth (Khan and Jinnah, 2002). In addition to the neurological symptoms, *lh* mice show reduced body weight and immunological problems when compared with unaffected litter mates (Sidman et al., 1965).

Since *lh* animals live to become adults, some type of VGCC must assume control of release of ACh at motor nerve terminals. Therefore, the objectives of the present study were to determine which subunits of the VGCCs are present at the motor nerve terminals of adult *lh* mice and control the release of Ach, and in these mice which β subunit(s) substitute for the β_4 subunit which normally co-associates with α_{1A}. Electrophysiological recordings of spontaneous and evoked release of ACh were made in *wt* and *lh* animals at intact NMJs in the presence of various VGCC antagonists. Additionally, the protein levels of different β subunits were compared in *lh* mice. To confirm our electrophysiological and protein assay results, immunostaining of cryosections of the *extensor digitorum longus* (EDL) muscle of *lh* and *wt* mice was done.

Materials and Methods:

Mice. Breeding pairs of heterozygoous Cacnb4lh4J mice were obtained from Jackson Laboratory (Bar Harbor, ME) and subsequently maintained in a breeding colony at Michigan State University Laboratory Animal Resources. Litters were genotyped at weaning, 3 wks after birth. *lh* (homozygous) mice were also identified by their characteristic phenotype consisting of a mild ataxia, wobbly gait behavior and smaller body size. Adult mice between 3 to 9 mos of age were used for all the experiments. All experiments were performed in accordance with local university (Michigan State University Laboratory Animal Resources) and national guidelines (National Institutes of Health of the USA - NIH) and were approved by the University Animal Use and Care Committee.

Drugs and chemicals. The presence of the various VGCCs was tested by the use of specific antagonists. To test for the presence of the L-type VGCC, the tissue was treated with 10 μM nimodipine (Nim. -Sigma-Aldrich, St. Louis, MO). The involvement of P/Q-type VGCC was determined with the use of 100 nM of ω-agatoxin IVA (Aga IVA - Alomone Labs, Jerusalem, Israel). The presence of the N-type VGCC was assessed with the use of 3 μM of ω-conotoxin GVIA (Ctx GVIA – Bachem California, Torrance, CA). The R-type VGCC was determined using 1 μM SNX 482 (Ascent Scientific, Princeton, NJ).

Antibodies against the various α_1 subunits were obtained from Alomone Labs (Jerusalem, Israel). To probe for the presence of the various β subunits we used different antibodies, for $\beta_{1,2}$ and $_4$ (Neuromab, UC Davis, University of California, CA) and β_3

(Santa Cruz Biotechnology Inc., Santa Cruz, CA) subunits. Fluorescein (FITC)-conjugated goat anti-rabbit IgG (heavy + light chains) was purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). Pacific Blue goat anti-mouse IgG (heavy + light chains) was obtained from Invitrogen (Carlsbad, CA) and tetramethylrhodamine α-bungarotoxin was purchased from Sigma (St. Louis, MO). All antibodies were used in a dilution of 1:200.

Electrophysiology. Animals were sacrificed by decapitation following anesthesia with 80% O_2 + 20% CO_2. The diaphragm with its attached phrenic nerves was removed from each animal and pinned in a Sylgard-coated chamber. The tissue was superfused continuously with 100% oxygenated physiological saline solution (137.5 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 11 mM *d*-glucose, and 4 mM HEPES, adjusted to pH 7.4). MEPPs and EPPs were recorded from *lh* and *wt* mice using conventional intracellular recording techniques. EPPs were elicited by phrenic nerve stimulation at 0.5 Hz using a suction electrode attached to a stimulus isolation unit (Grass SIU5; Grass Instruments, Quincy, MA) and stimulator (Grass S88). Signals were amplified using an Axoclamp-2 amplifier (Molecular Devices, Sunnyvale, CA), digitized using a PC-type computer and Axoscope 9.0 software (Molecular Devices), and analyzed using MiniAnalysis 6.0 Software (Synaptosoft, Decatur, GA). Recordings were performed from 5-10 end-plates for a period of 5 mins each for each mouse. Muscle action potentials were inhibited by addition of 2.5 μM μ-conotoxin GIIIB (Alomone Labs, Jerusalem, Israel). MEPPs and EPPs were recorded using intracellular glass microelectrodes (1.0 mm- o.d.; WPI, Sarasota, FL) which had a resistance between 5 and 15 MΩ, when filled

with 3M KCl. MEPP and EPP amplitudes were normalized to a membrane potential of -75 mV using the following formula: Vc = [Vo x (-75)]/E, where Vc is the corrected amplitude of MEPP and EPP, Vo is the actual recorded amplitude of MEPP and EPP and E is the membrane potential at which the recording is made (Magleby and Stevens, 1972; McLachlan and Martin, 1981). A recording was rejected if the 10-90% rise time was greater than 1.5 ms, also, if the membrane potential was more depolarized than -55 mV. The *m* value (quantal content) was calculated using the corrected mean amplitude values of EPP and MEPP, where *m* = EPP/MEPP (Hubbard et al., 1969).

Protein Isolation and Western Blot Analysis. Western blots were not sufficiently sensitive to detect the scarcity of VGCCs subunits in the presynaptic area of the diaphragm muscle. Therefore, I performed western blots to determine the protein levels in cerebellum of *lh* and *wt* mice. The protein level of α_{1A}, β_1 - β_4 subunits at *lh* and *wt* mice was compared by western blots of protein isolated from cerebellum which expresses of high concentrations of $Ca_V2.1$. The tissue sample was placed in a mortar containing 1 ml of 2X lysis buffer with 50 µl of each protease (20X stock solution of pepstatin, leupeptin, ethylenediaminetetraacetic acid (EDTA), ethylene glycol tetraacetic acid (EGTA) and protease inhibitors (Roche)). The tissue was ground, the lysate transferred to a microfuge tube, and centrifuged for 10 mins at 13000 RPM. The supernatant was stored at -80ºC. The protein concentration was determined using the bicinchoninic acid assay (BCA), and quantified with the Beckman Du 640 spectrophotometer (Beckman, Brea, CA). The proteins were loaded to a 10% SDS PAGE gel and migrated at a constant current of 40 mA. They were then transferred to a nitrocellulose membrane, this was

done at 4C and constant voltage of 90 mV. The membrane was probed against β-actin (dilution 1:20,000) as a loading control, and against α_{1A} (dilution 1:200) and β_{1-4} subunits (dilution 1:200).

Immunohistochemistry. *Extensor digitorum longus* (EDL) from *wt* and *lh* mice was used for immunohistochemistry. The muscle was dissected and fixed for 30 min in 4% (w/v) paraformaldehyde in 0.1M phosphate-buffered saline (PBS; composition 137 mM NaCl, 2.7 mM KCl, 1.4 mM NaH_2PO_4, and 4.3 mM Na_2HPO_4, pH 7.4). Then the tissue was washed in PBS for 1 min and treated with 0.1% (w/v) Triton X-100 in PBS for 30 min; after which the tissue was washed for 15 min with PBS and cryoprotected in 20% and 30% (w/v) sucrose each for 24 hs. The tissue was then placed in optimal cutting temperature compound (Tissue Tek, Tokyo, Japan) in a plastic mold and stored at -20ºC until used. Longitudinal sections (20 μm thick) were cut in a cryostat (Cryostat model Microm HM 525, Thermo Shandon Inc., Pittsburgh, PA) and mounted onto gelatin-coated slides. After this the tissue was stained with specific antibodies. α-Bungarotoxin was used as a marker for the ACh receptor in the muscle. The nerve was stained with antibodies against α_1 and β_{1-4} subunits. The preparations were viewed on a Nikon Eclipse TE 2000-U Diaphot-TMD microscope (Nikon, Melville, NY) with a Hamamatsu Orca 285 charge-coupled device camera (Bridgewater, NJ), and images were acquired using Metamorph software (Molecular Devices, Sunnyvale, CA).

Statistical Analysis. Differences between genotypes were analyzed using a one way analysis of variance followed by Tukey's test. Differences between treatments were analyzed using a two way analysis of variance followed by Tukey's test. *P* values were set to < 0.05 for all tests. Measurements are expressed as mean ± S.E.M for $n \geq 5$.

Results:

Effect of the *lh* mutation in cerebellar protein levels

Since *lh* animals lack the β_4 subunit, we first wanted to determine whether this mutation affected the protein levels of the remaining different β subunits of adult *lh* mice. We first tried to assay for protein level in diaphragm and phrenic nerve. However, due to the scarcity of VGCC proteins present in this tissue and the poor sensitivity of western blots we were unable to probe against any subunit (no bands were revealed in these blots, Fig. 3.1a). Next we probed for the different VGCC subunits in cerebellum. We chose this tissue since the β_4 subunit is normally extensively expressed in cerebellum and this region of the brain is responsible for motor coordination. *Lh* mice exhibit poor motor coordination and balance, indicating that cerebellar dysfunction likely occurs. Because the β_4 subunit normally co-associates with α_{1A}, we also wanted to assay for α_{1A} levels to determine if the absence of β_4 affects the levels of α_{1A}. Protein level analysis, through western blot assays showed that even though *lh* animals lack the β_4 subunit, the protein levels of the α_{1A} subunit are the same for *lh* and *wt* animals. This result may be due to the observed significant increased levels of β_1 and β_3, which might compensate for the lack of β_4 (Fig.3.1 b and c).

ACh release is controlled by P/Q- and R-type VGCCs in adult *lh* mice

According to the western blot results, the *lh* mutation appeared not to affect the α_{1A} protein levels as compared to their *wt* littermates. However, these mice are

apparently compensating for the absence of β_4 with increased levels β_1 and β_3. Based on these results, we wanted to determine if this atypical association affected the types of VGCC being expressed in NMJs of adult *lh* mice. For this purpose we performed intracellular recordings in the diaphragms of adult *lh* and *wt* mice, in the presence of different VGCC antagonists.

ACh release following phrenic nerve stimulation is significantly decreased both in *lh* as well as *wt* animals (60% and 76%, respectively) after treating the tissue with ω-agatoxin IVA (ω-Aga IVA; 100 nM), a specific antagonist of the P/Q-type VGCC. The difference between both genotypes was not statistically significant. We therefore wanted to test whether other types of VGCC are present and contribute to the release of ACh in *lh* mice. The antagonist of the R-type VGCC, SNX 482 (1 μM), significantly decreased the quantal content of *lh* (46%), but not *wt* mice (8%). This implied that R-type VGCCs contribute to the control of the ACh release in the NMJ of adult *lh* but not in *wt* mice.

In contrast, the contribution of L- and N-type VGCCs to the control of ACh release both in *wt* and *lh* mice was negligible. ω-Conotoxin GVIA (ω-ctx GVIA) (3 μM) a specific antagonist of the N-type VGCC, did not significantly change the quantal content of either *lh* or *wt* mice. The same result occurred when nimodipine (Nim) (10 μM) was applied to test for the presence of L-type VGCC. Nimodipine did not induce any change in the ACh release in either *lh* or *wt* mice. These results imply that L- and N-type VGCCs do not contribute significantly to control ACh release at the NMJ of adult *lh* or *wt* mice.

When we co-applied ω-Aga IVA and SNX 482 they significantly reduced ACh release in *lh* to a level similar to that observed after the application of ω-Aga IVA alone

in *wt* mice. Taken together, these results implicate that in *lh* animals ACh release is controlled by P/Q- and R- type VGCCs, and that both types contribute to a similar extent (Fig.3.2). However this is not the case in *wt* mice, since when we combined the two antagonists we saw no significant change from the results obtained following application of ω-Aga IVA alone (Fig.3.3).

Additionally as a control, we applied Cd^{2+} (10 μM), a non-specific blocker of VGCCs, to some preparations. This concentration of Cd^{2+} effectively blocked ACh release in both *wt* and *lh* mice (data not shown).

Immunohistochemistry data

Western blot results showed an increased level of β_1 and β_3 subunits, while electrophysiological results indicated that P/Q- and R-type VGCCs control ACh release at NMJs. Consequently, we wanted to determine the presence of the different α_1 and β subunits at NMJs of adult mice. The localization of the different α_1 and β subunits at *lh* and *wt* mice NMJ was examined using fluorescence immunohistochemistry in sections of *extensor digitorum longus* (EDL) muscle. The sections were stained with specific antibodies against the various α_1 and β subunits as well as the muscle type nACh receptor (as a postsynaptic marker).

As demonstrated by the representative immunostaining images (Fig.3.4) *wt* animals have staining of α_{1A} (green), β_4 (blue), and α-bungarotoxin (red); adult *lh* mice

have no immunostaining of β_4, but there is immunostaining of β_3, β_1 (blue), α_{1A}, α_{1E} (green), and α-bungarotoxin (red) (Figs.3.5 and 3.6).

Figure 3.6 compares the relative fluorescence for each subunit in both genotypes. There is a significant increase in α_{1E}, β_3, and β_1 in *lh* mice as compared to their *wt* littermates. However the levels of α_{1A} in *lh* mice are slightly decreased as compared to *wt*, yet this change is not significantly different ($p>0.05$). This is consistent with the results of both western blot and electrophysiology. We also quantified the extent of juxtaposition of the VGCC subunits against the nACh receptor present in both genotypes (Fig. 3.7). In *lh* mice there is a 58% and 44% juxtaposition of α_{1A} and α_{1E} respectively, while in *wt* there was 73% juxtaposition of α_{1A} with α-bungarotoxin. With regards to the β subunits, in *lh* mice β_1 and β_3 juxtaposed with the nACh receptor at 88% and 65%, respectively.

The immunohistochemistry data confirmed the electrophysiology data; both α_{1A} and α_{1E} staining overlaps that of nACh receptors at the NMJ. We were also able to determine that there is an increase in β_3 and β_1 subunit in *lh* mice, which might compensate for the absence of β_4 subunit.

Discussion:

In normal adult mammals, ACh release is mainly controlled by P/Q-type VGCCs (Katz et al., 1995). We know that α_{1A} knockout mice do not survive more than 15-21 days after birth (Jun et al., 1999). We also know that under specific conditions, different subtypes of VGCCs can mediate ACh release when they would not do so normally, such as in *tg* mice (Flink and Atchison, 2002; Pardo et al., 2006). The β subunit regulates the assembly and membrane localization of the α_1-pore forming subunit of the VGCCs (Walker and De Waard, 1998). β_4 subunits normally coassociate with α_{1A} pore forming subunit of the neuronal P/Q-type VGCCs (Burgess et al., 1997; Catterall, 1995). Based on this information we hypothesized that *lh* mice might present compensation by other types of VGCC due to the absence of the β_4 subunit. These mice live to adulthood, and are fertile. Moreover, they show no obvious neuromuscular impairment.

Our results show the following: (1) Even though *lh* animals lack the β_4 subunit, levels of the α_{1A} subunit are similar for *lh* and *wt* animals. This may be due to the observed increased level of β_1 and β_3, to compensate for the lack of the β_4 subunit. (2) ACh release following nerve stimulation is significantly decreased both in *lh* as well as *wt* animals after treating the tissue with ω-Aga IVA, a specific antagonist of P/Q-type VGCCs. (3) ACh release following nerve stimulation is significantly decreased in *lh* animals after treating the tissue with SNX 482, a specific antagonist of R-type VGCCs. (4) In *lh* mice β_1 and β_3 subunits seem to associate with α_{1A} and α_{1E}.

Unlike the *tg* mouse for which a direct mutation occurs in α_{1A} (Pardo et al., 2006), the *lh* mouse maintains a functional complement of P/Q-type VGCCs and association of β_4 with α_{1A} is not obligatory for functional expression of the protein, albeit at apparently reduced abundance.

The effect of ω-Aga IVA on *wt* and *lh* NMJ was not significantly different, however it did show an interesting trend in which *wt* mice showed inhibition of 76% after application of the P/Q-type antagonist, while in *lh* the same protocol produced 60% inhibition. It was this difference which led us to think that other VGCCs might be present at the NMJ of adult *lh* mice.

The significantly differential effect of SNX 482 on *wt* and *lh* mice indicates that ACh release in adult *lh* NMJ is controlled in part by R-type VGCC, while this subtype is not involved in ACh release at NMJ of adult *wt* mice.

Increased immunofluorescence of α_{1E} at NMJs of EDLs in *lh* animals, confirmed the electrophysiological results showing the involvement of the R-type VGCC. We also saw similar levels of fluorescence in *wt* and *lh* mice in the α_{1A} subunit, which also implies that there are P/Q-type VGCCs present at the NMJ. With regards to the auxiliary cytoplasmic β subunit, we saw an increase in fluorescence of the β_1 and β_3 in *lh* mice, which may compensate for the absence of the β_4 subunit.

Although this work shows some results that are similar to those presented by Kaja (2007), most of the results presented in this work differ from his. Kaja et al. (2007) determine that P/Q-type VGCCs are the only type of channel controlling ACh release at

adult *lh* NMJ. The difference between the results of the two studies could reside in the age difference between the animals used. While he performed his entire study in 6 wk old mice, we used 3-9 mo old mice for our studies. At the comparatively young age of mice at which Kaja (2007) examined ACh release, there might have been a greater dependence on P/Q-type and a lesser contribution of R-type channels than in 3 to 9 mo old animals. Additionally there are slight differences in the methodology used between us. These differences could possibly lead to differences in our results. This would need to be examined further, by repeating the experiments using his protocol.

In conclusion, the type of channel that can control ACh release at mammalian NMJ is not fixed. It has been shown that recruitment of alternative types of VGCCs to compensate for a deficit is possible. For example in *tg* mice, which carry a point mutation in the α_{1A} subunit of the P/Q-type VGCCs compromising the channel's function, ACh release is controlled by N- and R- type VGCCs (Pardo et al., 2006). Therefore, it is not unlikely that in *lh* mice, there is a form of neuronal plasticity in ACh release to compensate for their mutation.

Figure 3.1: Effect of *lh* mutation on protein levels.

Western blots were performed to probe for protein levels of the different VGCCs subunits. β-Actin was used as a loading control. a) Representative western blot of VGCCs subunits present in diaphragm and phrenic nerve from *wt* mice. The tissue was loaded in the following order: (1) diaphragm (40 µg), (2) diaphragm (60 µg), (3) phrenic nerve (40 µg), (4) phrenic nerve (60 µg). Protein levels from *wt* mice preparations were probed with antibodies for α_{1A} and $\beta_{1\text{-}4}$. The western blots did not reveal any VGCCs subunit in these tissues, but was sensitive to β-Actin, which was used as a loading control. (b) Representative western blot of VGCCs subunits present in cerebellar proteins (40 µg). Protein levels from *lh* and *wt* cerebellar preparations were probed with antibodies for α_{1A} and $\beta_{1\text{-}4}$. (c) Protein levels were quantified using the program Image J®. Each value represents the mean ± S.E.M of 7 animals. The asterisk (*) indicates a significant difference between the two genotypes.

Figure 3.1 (cont'd)

(a)

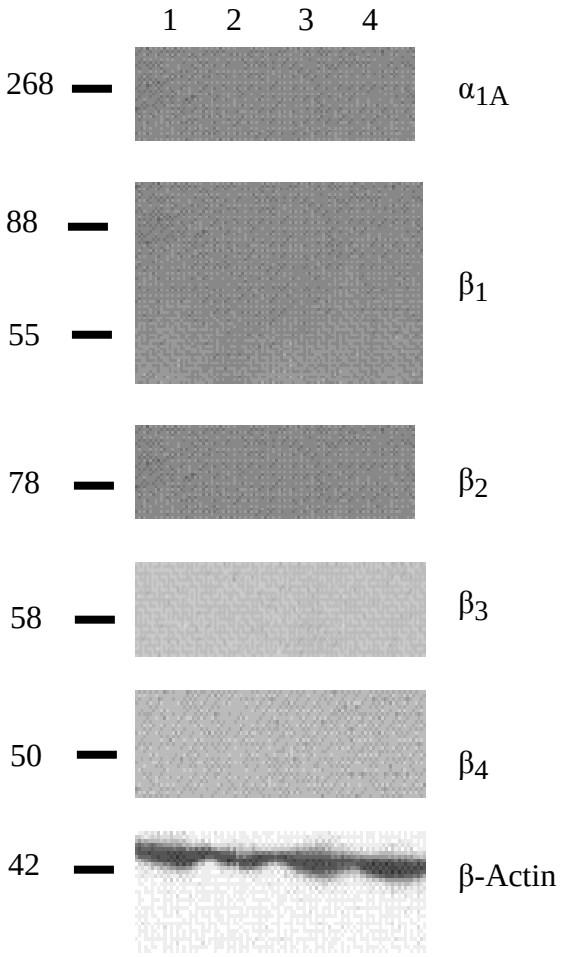


Figure 3.1 (cont'd)

(b)

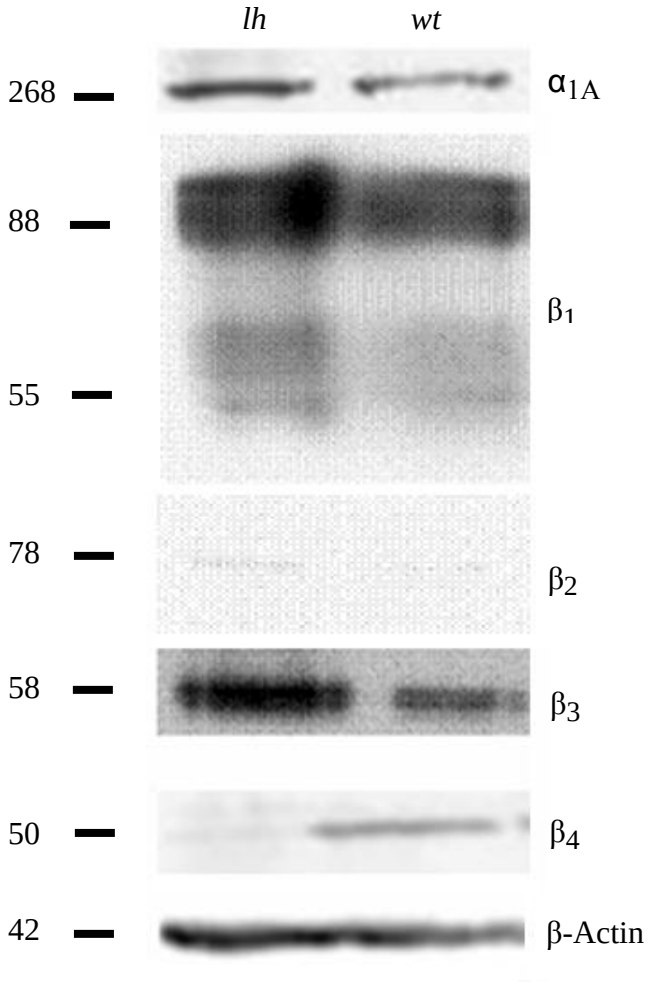


Figure 3.1 (cont'd)

(c)

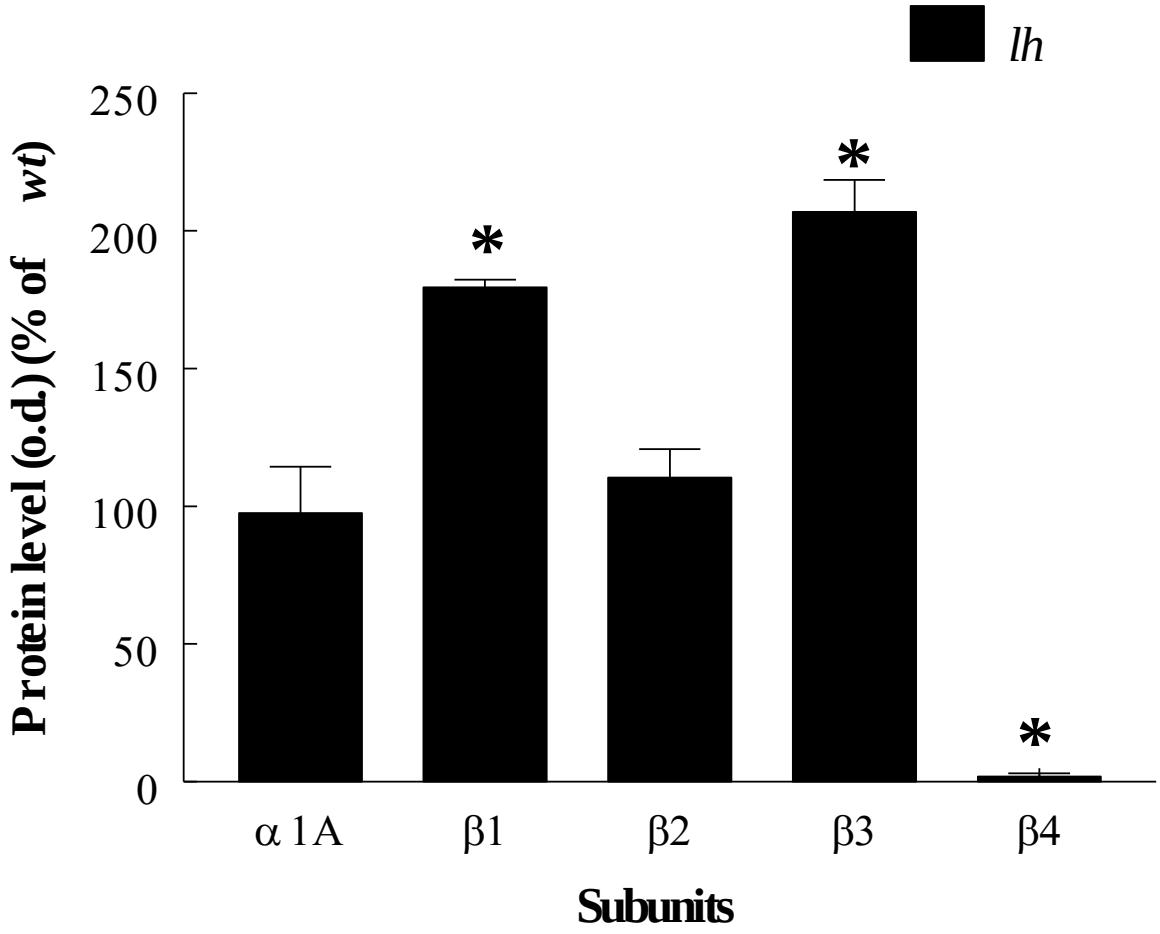


Figure 3.2: ACh release is controlled by P/Q- and R-type VGCCs in adult *lh* mice.

Effect of VGCC antagonists on nerve-evoked ACh release from motor nerve terminals of adult *lh* and *wt* mice. The tissue of *wt* or *lh* mice was treated with VGCC antagonists. (a - c) Shows EPPs from neuromuscular junction preparations isolated from homozygote lethargic (*lh*) and *wt* mice with no pharmacological treatment (black control trace) or treated by incubation with ω-Aga IVA (orange trace), ω-Ctx GVIA (light blue trace), Nimodipine (purple trace) or SNX 482 (green trace). (d) 100 nM of ω-Aga IVA reduced the quantal content in *lh* animals to 40%, while the same concentration reduced the quantal content in *wt* mice to 24%. This difference in the reduction level suggests the possible presence of other VGCC controlling the ACh release in *lh* mice. The application of ω-Cntx GVIA and nimodipine did not significantly changed the quantal content of either *wt* or *lh* mice. The use of SNX 482 significantly reduced the quantal content in *lh* animals, but not in *wt* mice. The asterisk (*) indicates significant difference from the control pretreated preparation. The number sign (#) indicates a significant difference between genotypes. Data represent the mean ± S.E.M. ($n \geq 8$).

Figure 3.2 (cont'd)

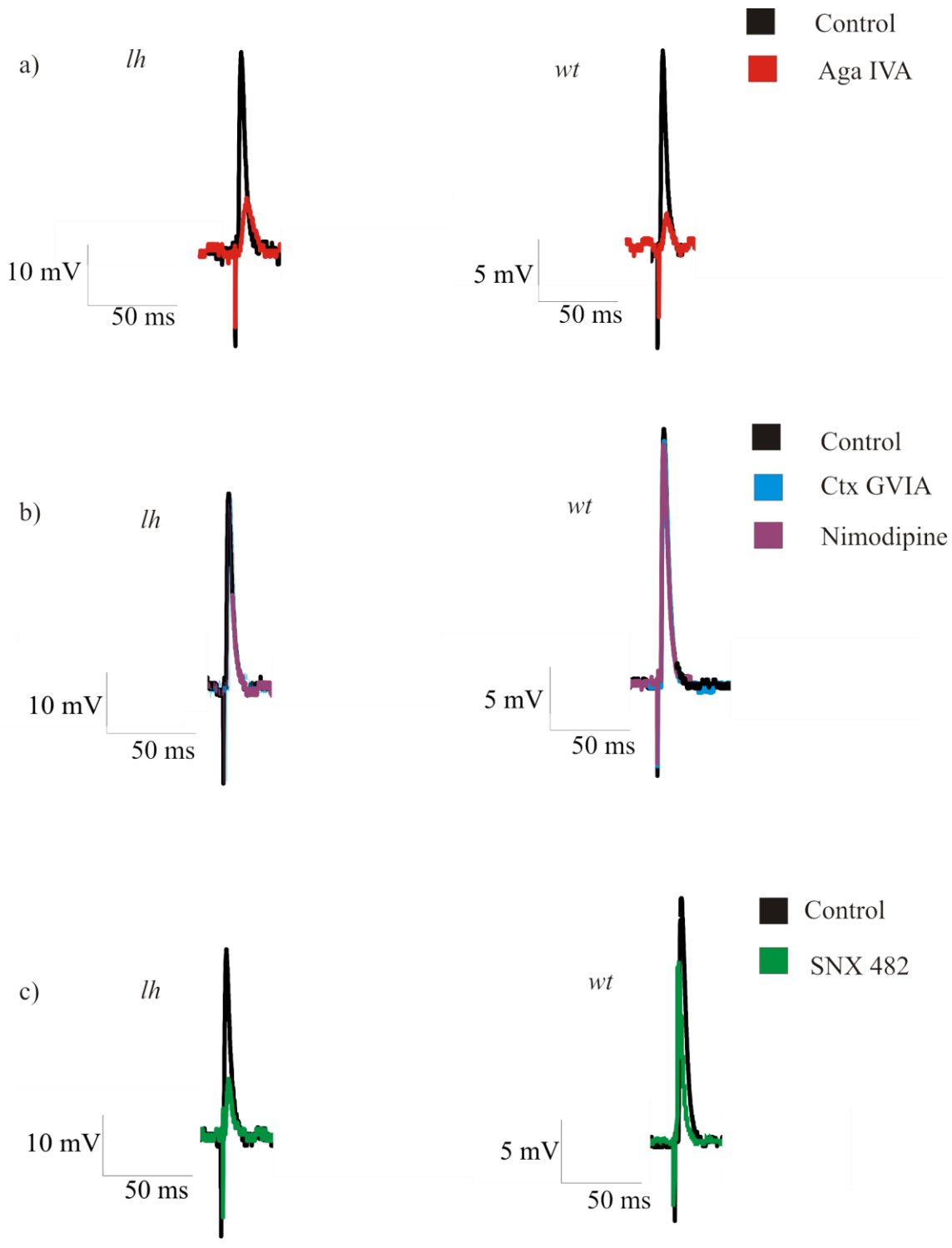


Figure 3.2 (cont'd)

(d)

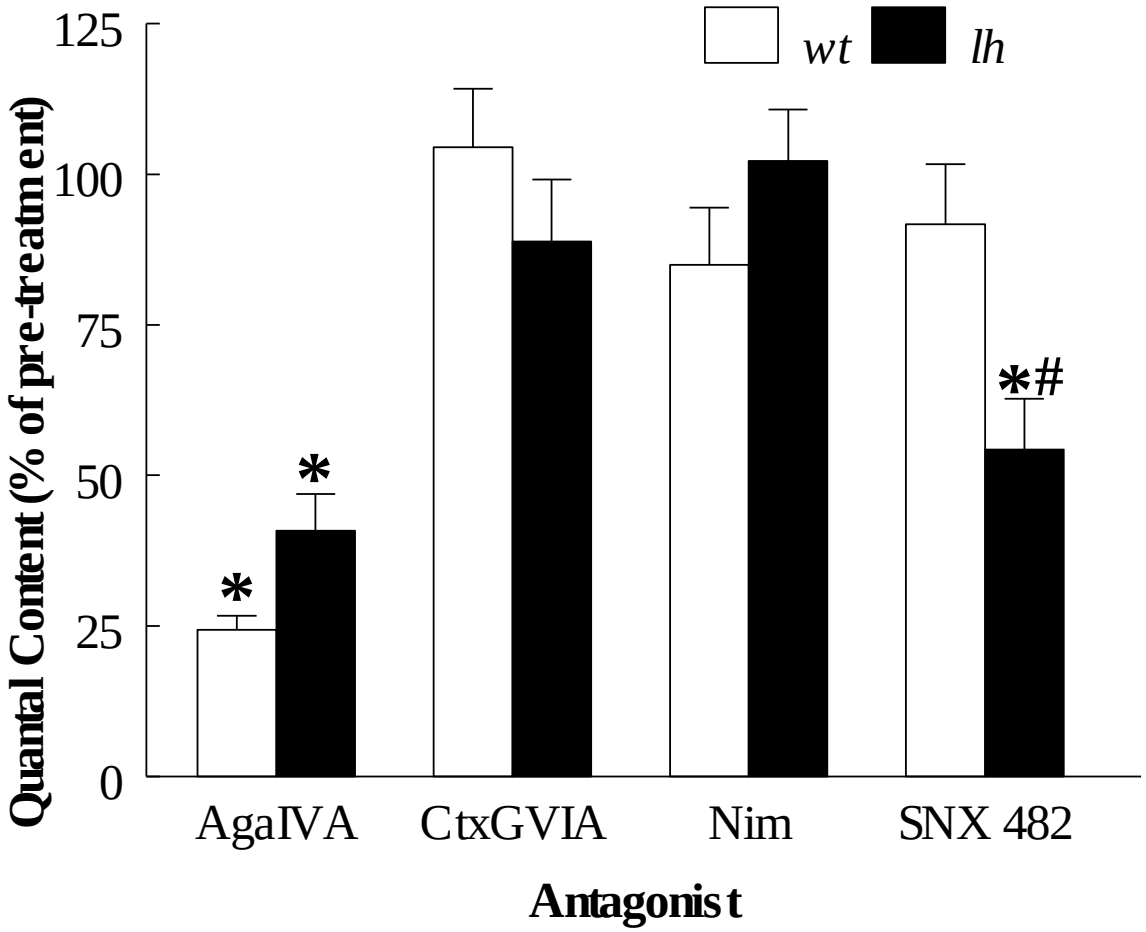


Figure 3.3: Effect of Aga IVA and SNX-482 on quantal content of *lh* and *wt* mice.

EPPs were recorded from NMJ preparations of adult *lh* and *wt* mice. SNX 482 and ω-Aga IVA were applied simultaneously to measure the combined effect of both antagonists. The application of both toxins in *lh* animals had a similar effect to that observed in *wt* with ω-Aga IVA alone. There was no significant change in preparations from *wt* mice when SNX 482 and ω-Aga IVA were applied together or when ω-Aga IVA was applied alone. However when both antagonists were applied together there was a further decrease in the quantal content in *lh* preparations as compared to that obtained when ω-Aga IVA or SNX 482 were applied alone. The asterisk (*) indicates a significant difference from the control pretreated preparations. The number sign (#) indicates a significant difference between genotypes. Data represent the mean ± S.E.M. ($n \geq 5$).

Figure 3.3 (cont'd)

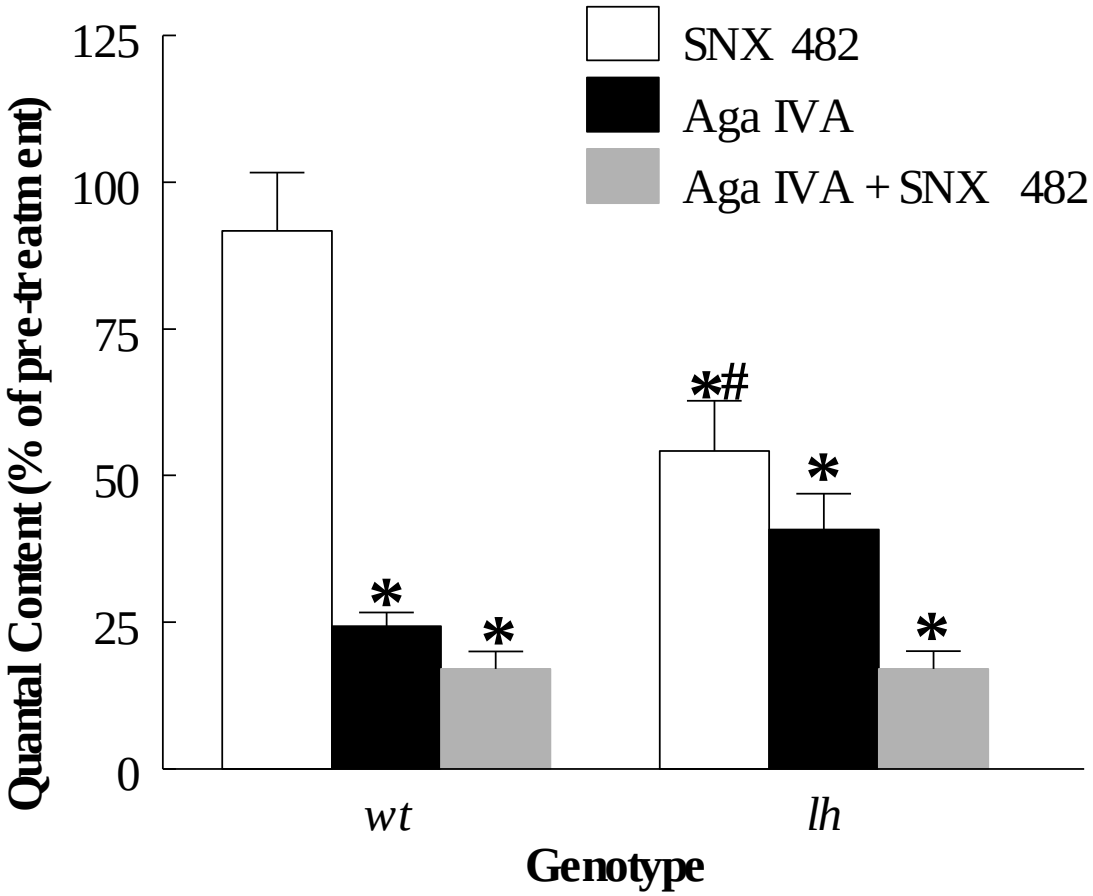


Figure 3.4: Immunostaining of *wt* and *lh* neuromuscular junction with α_{1A}/α_{1E} and β_4.

EDL muscles from *wt* and *lh* mice were stained with specific antibodies against VGCCs α_1 (green) and β_4 (blue) subunits as well as α-bungarotoxin (red). The tissue was then observed under the Nikon Eclipse TE 2000-U fluorescent microscope. The composite shows the superimposition of the various subunits. The bar scale is 10 µm.

Figure 3.4 (cont'd)

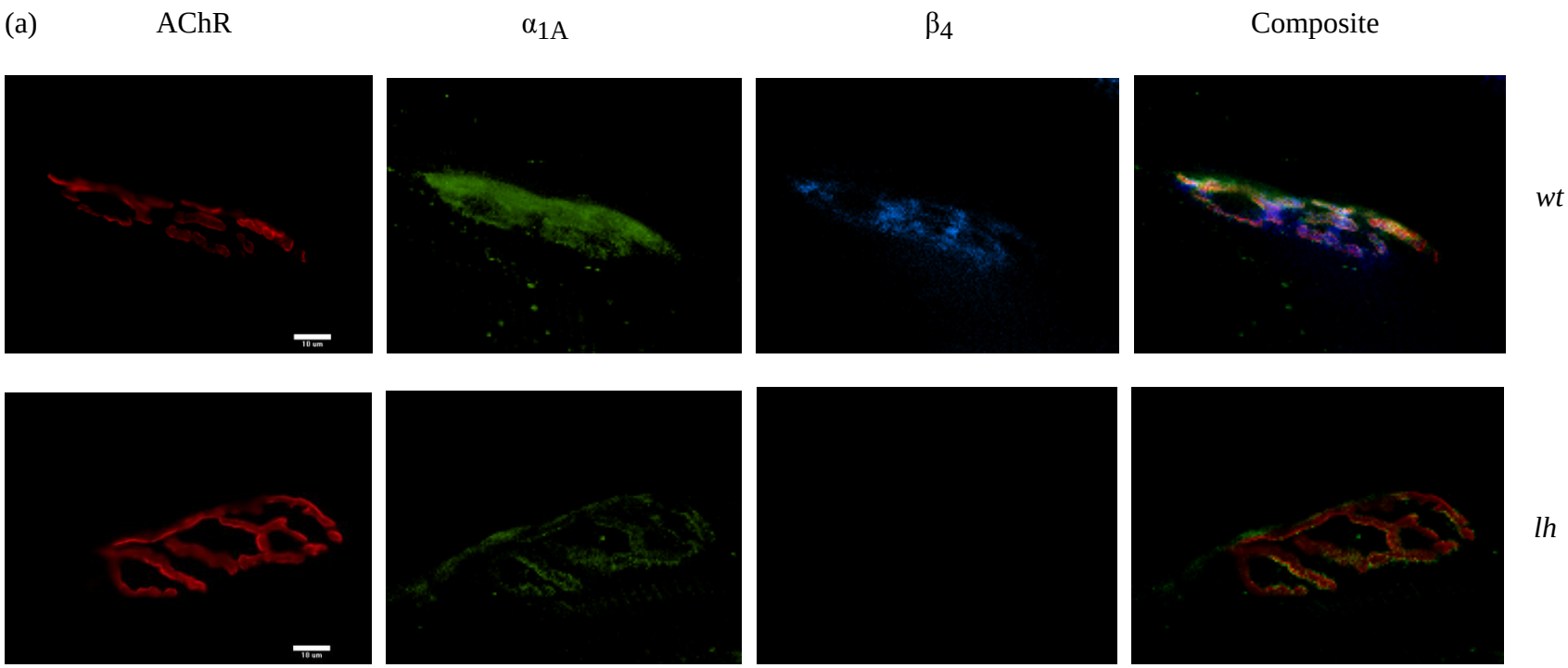


Figure 3.4 (cont'd)

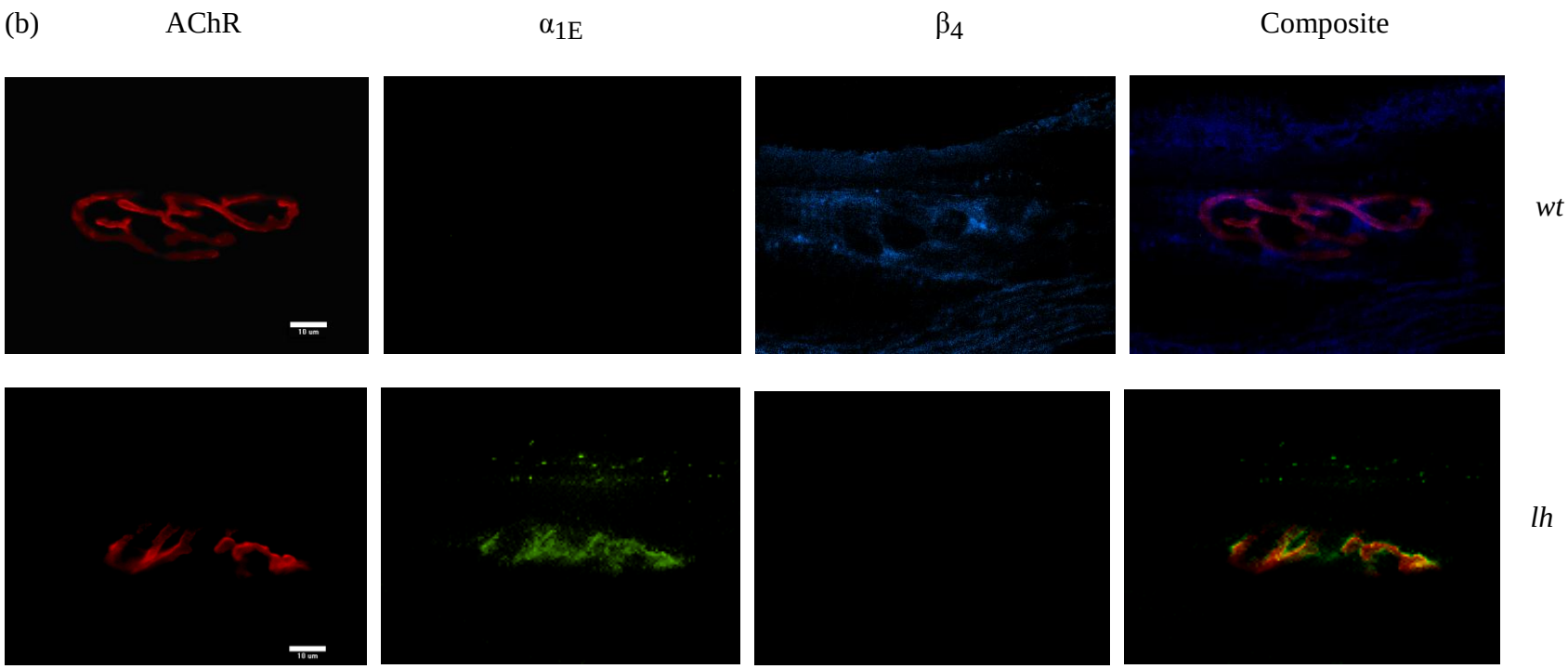
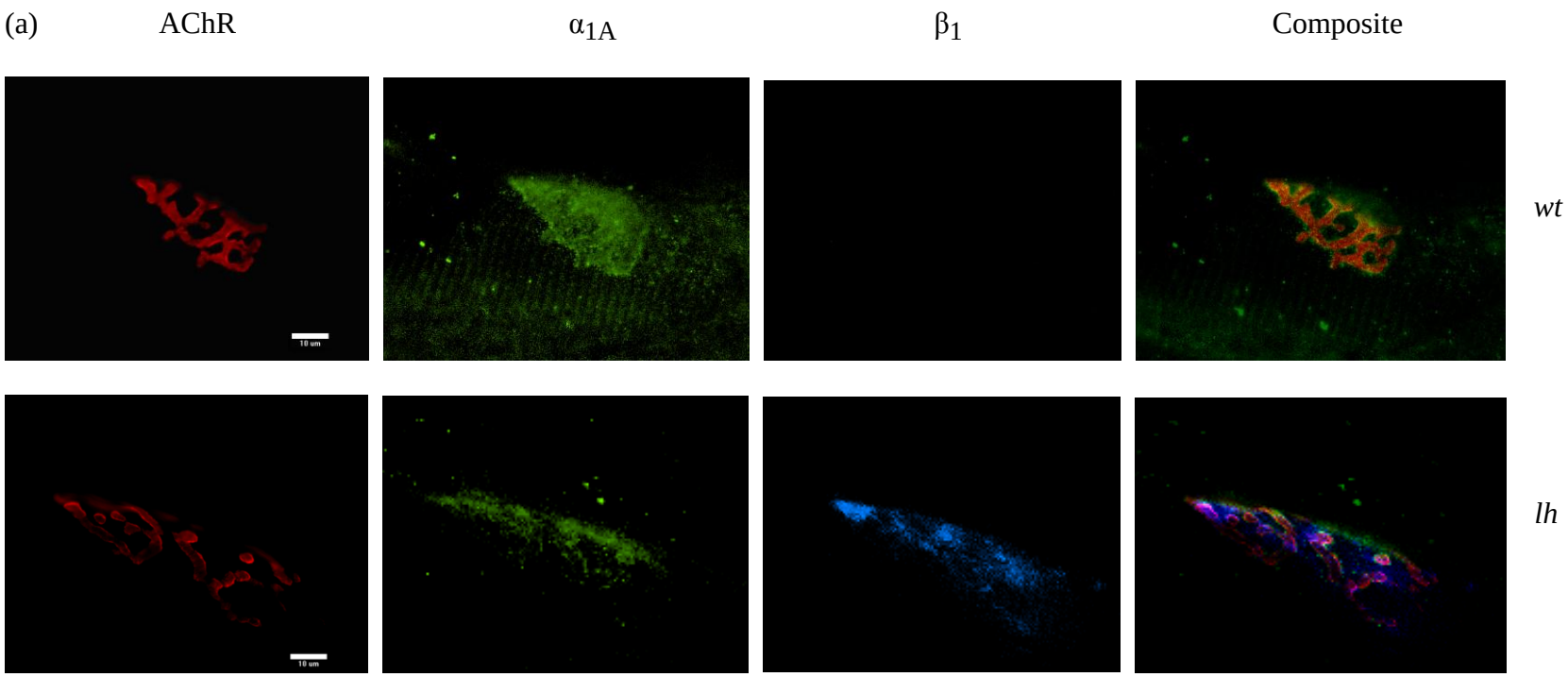


Figure 3.5: Immunostaining of *wt* and *lh* neuromuscular junction with α_{1A}/α_{1E} and β_1.

EDL muscles from *wt* and *lh* mice were stained with specific antibodies against VGCCs α_1 (green) and β_1 (blue) subunits as well as α-bungarotoxin (red). The tissue was then observed under the Nikon Eclipse TE 2000-U fluorescent microscope. The composite shows the superimposition of the various subunits. The bar scale is 10 μm.

Figure 3.5 (cont'd)



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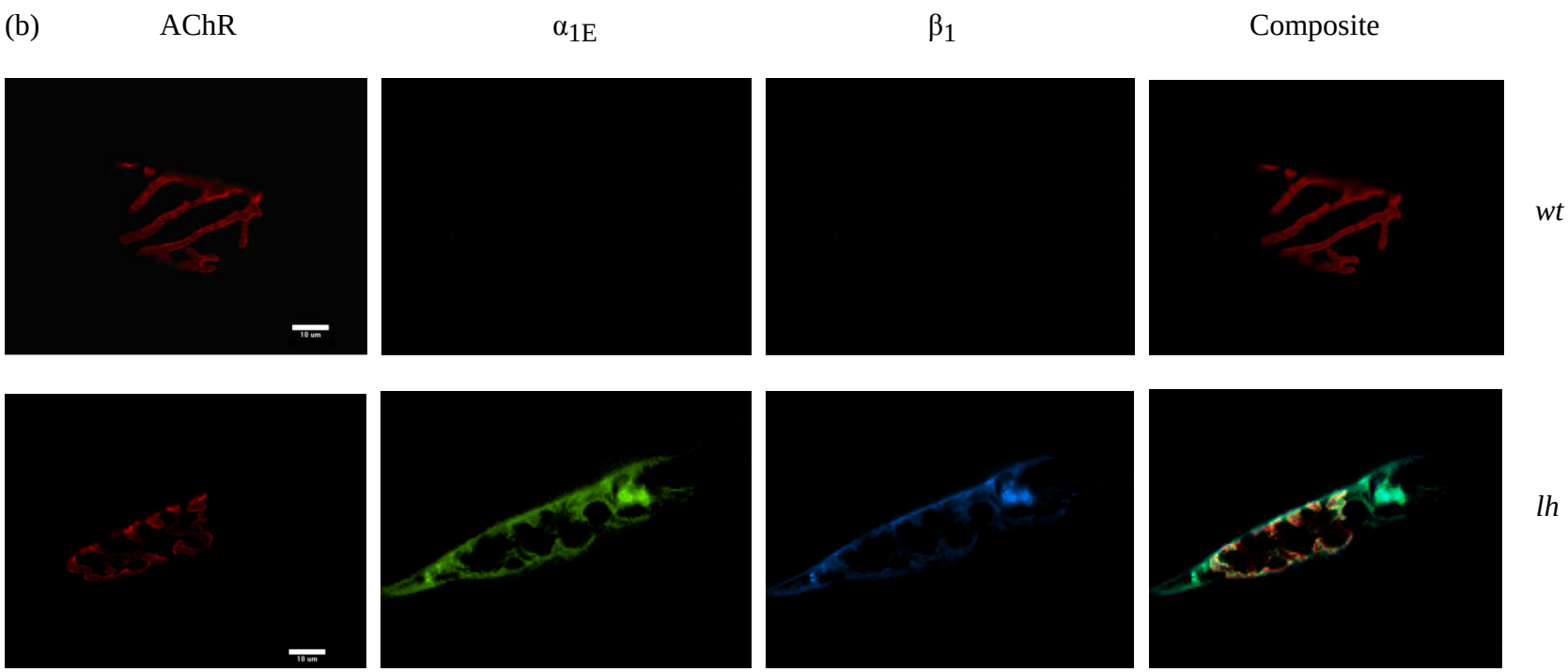


Figure 3.6: Immunostaining of *wt* and *lh* neuromuscular junction with α_{1A}/α_{1E} and β_3.

EDL muscles from *wt* and *lh* mice were stained with specific antibodies against VGCCs α_1 (green) and β_3 (blue) subunits as well as α-bungarotoxin (red). The tissue was then observed under the Nikon Eclipse TE 2000-U fluorescent microscope. The composite shows the superimposition of the various subunits. The bar scale is 10 µm.

Figure 3.6 (cont'd)

(a) AChR α_{1A} β_3 Composite

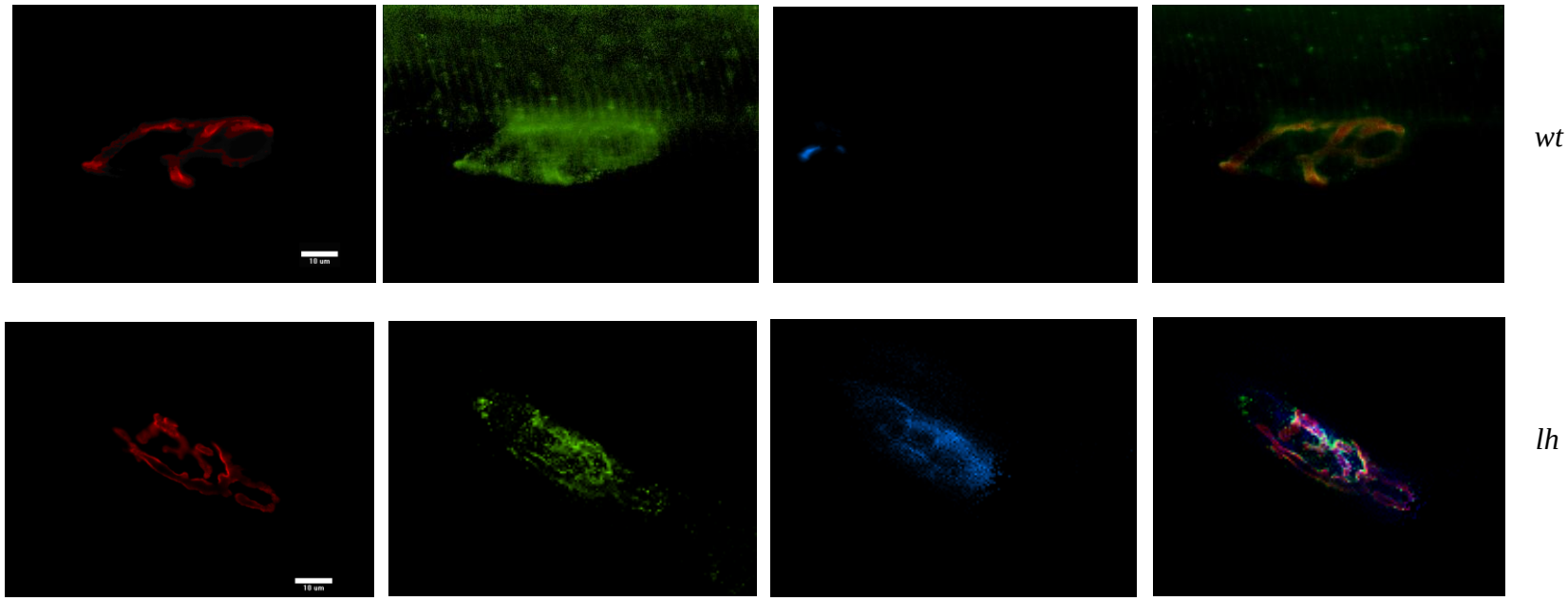
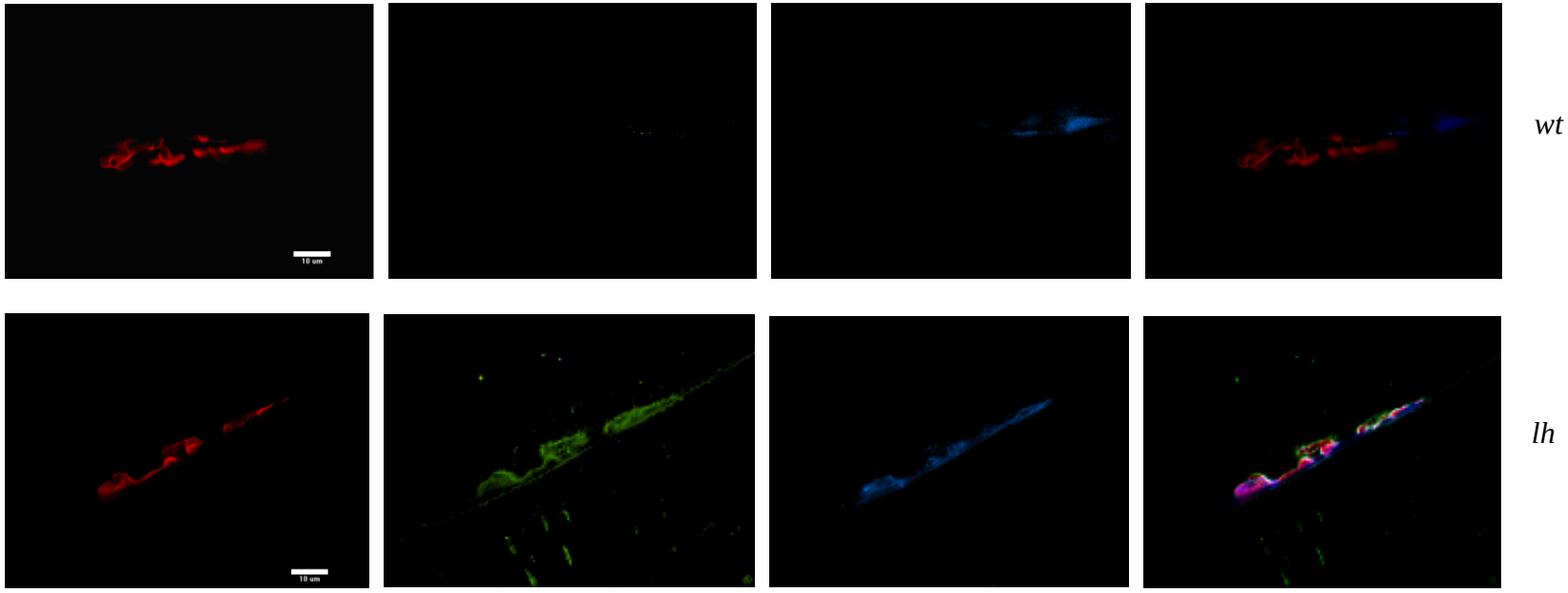


Figure 3.6 (cont'd)

(b) AChR α_{1E} β_3 Composite



wt

lh

Figure 3.7: Relative fluorescence of α_1 and β subunits at *wt* and *lh* NMJs.

Relative pixel count of fluorescence corresponding to α_1 and β subunits at *wt* and *lh* NMJ. EDL preparations were probed with antibodies against the specific VGCCs subunits. The fluorescence levels were quantified using MetaMorph®. The asterisk (*) indicates a significant difference from *wt* preparations. (n = 8).

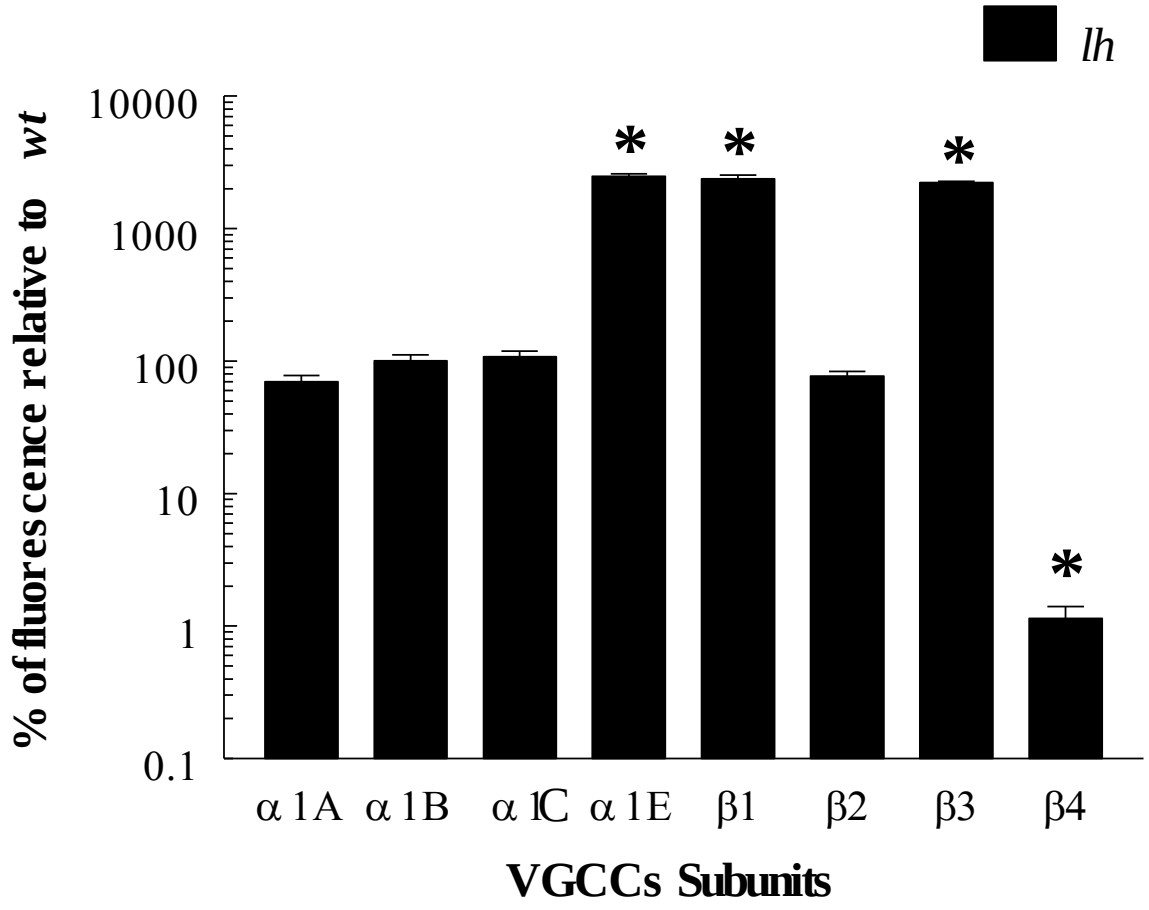
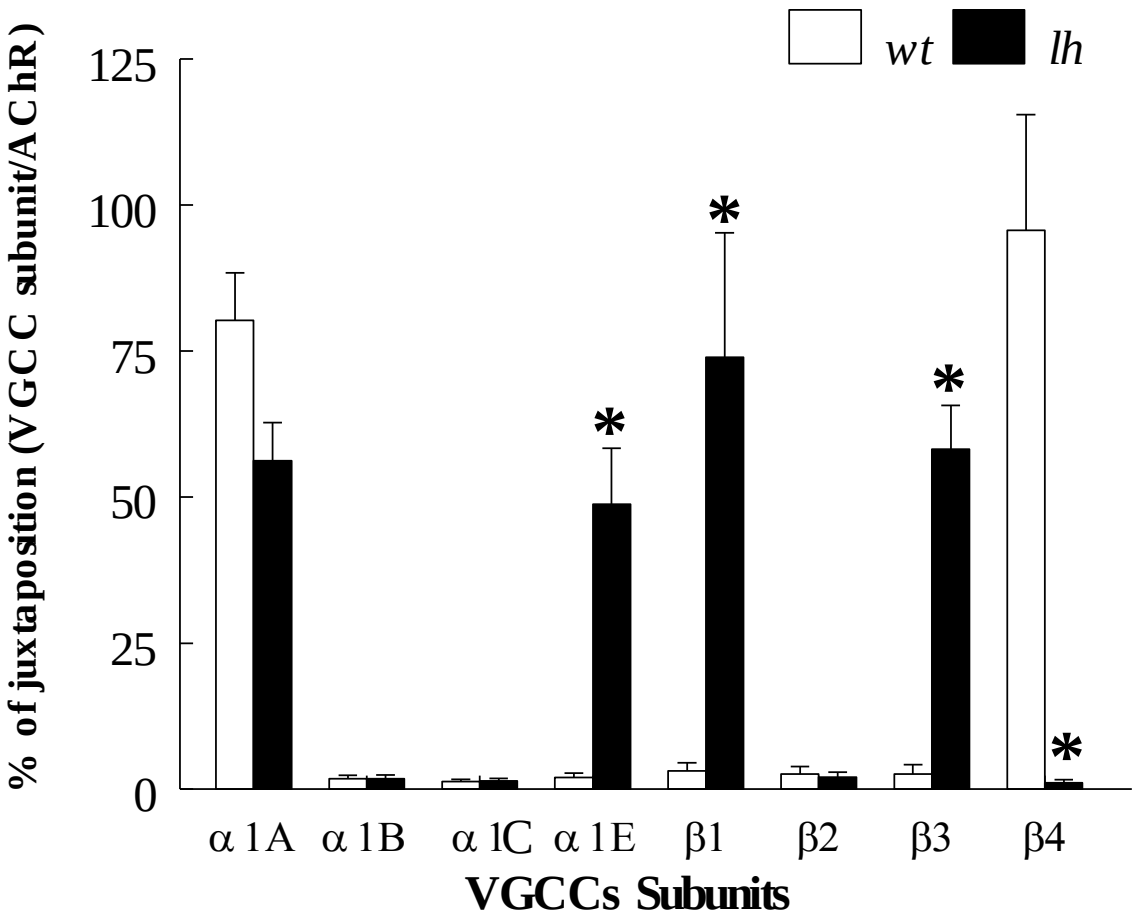


Figure 3.8: Percent juxtaposition of α_1 and β subunits of *wt* and *lh* NMJ with ACh receptors.

Percent juxtaposition of α-bungarotoxin and various subunits of VGCC in *lh* and *wt* NMJ. The values are taken from the data for the NMJ samples depicted in Fig. 3.7. The asterisk (*) indicates a significant difference from *wt* preparations. (n = 8).



CHAPTER 4

SUMMARY AND CONCLUSIONS

Summary and Conclusions

The overall aim of this dissertation was to examine acetylcholine (ACh) release in *lethargic* (*lh*) mice, and determine what effect disruption of the β_4 subunit has on the function of the α_{1A} subunit in *lh* mice. We know that the α_{1A} subunit is vital to the survival of adult mammals; α_{1A} knockout mice do not live past 2 weeks of age (Jun et al., 1999). Normally α_{1A} combines with β_4 to form functional P/Q-type channels. *Lethargic* mice live to adulthood, therefore these mice must compensate for the absence of β_4 either (1) by having a different voltage gated calcium channel (VGCC) controlling the ACh release (as is the case in the *tottering* (*tg*) mouse mutation), and/or (2) by substituting a different β subunit for β_4.

My dissertation's work has shown that even though the *lh* mutation does not affect miniature end-plate potential (MEPP) amplitude, it does affect the frequency of spontaneous release of ACh when induced by Sr^{2+} or Ba^{2+}, but not by Ca^{2+}. Additionally, it alters the amplitude of the nerve evoked ACh release, which translates to a decrease in quantal content. Moreover, the vesicle release process is slower in *lh* mice, as shown by the FM1-43 staining experiments. However when release is induced by hypertonic solution or α-latrotoxin (α-LTx), FM1-43 destaining is the same in both *lh* and *wild-type* (*wt*) mice. Even though *lh* mice lack the β_4 subunit, the protein levels of the α_{1A} subunit in cerebellum are similar to *wt* animals. This may be due to the observed increased levels of β_1 and β_3, which might compensate for the lack of β_4 subunit. To test

which VGCCs are controlling ACh release, intracellular recordings were performed in the presence of various VGCC antagonists. In adult *lh* mice, ACh release is controled by P/Q- as well as R-type VGCCs at the motor nerve terminal. This was corroborated by the immunohistochemistry results showing that there is an increased level of fluorescence of $\alpha_{1E,}$ but the levels of α_{1A} remained unaltered by this mutation, and there is an increase in β_1 and β_3 in the motor nerve terminal of *extensor digitorum longus* (EDL) muscle to compensate for the absence of β_4 subunit (Fig.3.7). In *lh* animals neurologic signs occur despite the normal level of α_{1A}. This might be due to the abnormal association in the *lh* animals of α_{1A}- β_1 or α_{1A}-$\beta_{3,}$ and the presence of α_{1E}- β_1 or α_{1E}-$\beta_{3,}$ which might affect the Ca^{2+} current.

VGCCs regulate diverse neuronal functions by mediating the entry of Ca^{2+} into nerve terminals. VGCCs are multiple subunit structures which contain the α_1 subunit - the major pore-forming subunit, and several auxiliary subunits - $\alpha_2\delta$, β and γ (Catterall, 1995, 2000; Hoffmann et al., 1999; Zhang et al., 1993). The β subunits are cytoplasmic proteins. They regulate the assembly and membrane localization of the α_1 subunits, influence the current amplitude, rate, activation/inactivation kinetics, and ligand-binding sites on motor nerve VGCCs (Brice and Dolphin, 1999; Catterall, 1995; Walker and De Waard, 1998). There are four different types of β subunits ($\beta_{1\text{-}4}$) each encoded by different genes (Chien et al., 1995). There are different types of VGCCs which can be classified according to their pharmacological and physical properties. In mature

mammalian motor nerve terminals, it is the P/Q-type calcium channels that control ACh release (Katz et al., 1995). However the complement of VGCCs is not necessarily fixed. Under specific conditions, subtypes of VGCC that are not normally associated with the ACh release at motor nerve terminals can mediate it. Lambert-Eaton Myasthenic syndrome (LEMS) is a paraneoplastic disorder, in which autoantibodies target the α_{1A} subunit of the P/Q-type VGCC. When *wt* mice are induced to show LEMS like signs by repeated administration of plasma from LEMS patients, these mice showed that L-type VGCCs were contributing to ACh release from the motor nerve terminal (Flink and Atchison, 2002). Moreover, *tg* mice have a point mutation in the α_{1A} subunit of the P/Q-type VGCC. This causes *tg* mice to show an ataxic phenotype. In this case, *tg* mice compensate for the mutation in the P/Q-type VGCC, by controlling ACh release by N- and R-type VGCCs. Recruitment of alternate types of VGCCs at motor nerve terminals to control neurotransmitter release is a frequent method of neuronal plasticity (Pardo et al., 2006).

The β_4 subunit, which is widely expressed in the brain (Burgess et al., 1997; Catterall, 1995), is normally associated with the α_{1A} subunit of the neuronal P/Q- VGCC (Witterman et al., 2000). There is a naturally-occurring mutation that leads to the *lh* mutant mouse. There is a four bp insertion into a splice donor site, in mouse chromosome 2 (Fig.1.8), which results in exon skipping, translational frameshift, and protein truncation. Reduced levels of transcripts are produced from this allele and are present at approximately 20% of the normal levels in homozygous mice (Burgess et al., 1997). The β_4 subunit of these animals is missing 60% of the C-terminal region which includes the

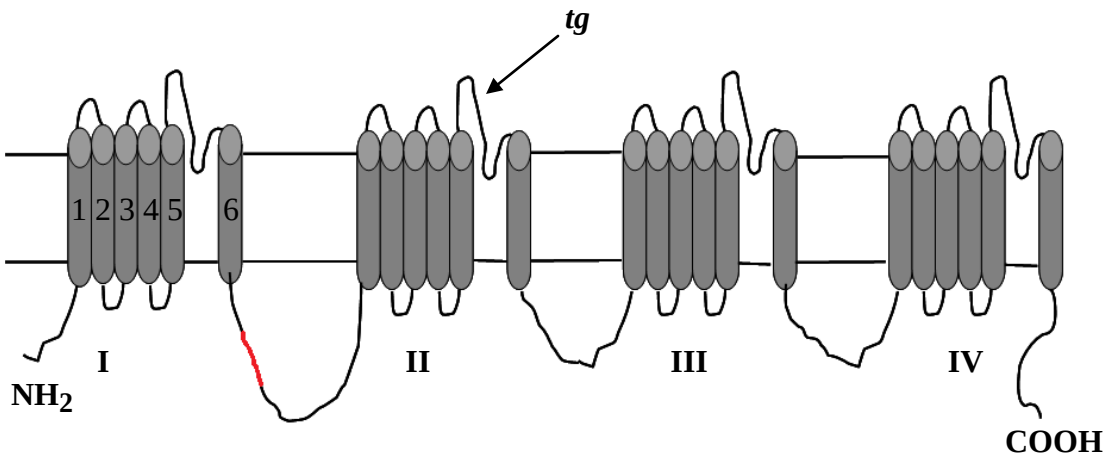
α_1-binding site, suggesting that this defect in calcium channel assembly could be one cause for the pathogenesis in *lh* phenotype (Burgess et al., 1997; Burgess and Noebels, 1999). Mice with this mutation have a very distinct phenotype that becomes apparent at 15 days of age (Khan and Jinnah, 2002). They suffer from ataxia, lethargic behavior, spike-wave epilepsy and paroxysomal dyskinesia, reduced body weight and immunological problems, when compared with unaffected litter mates (Sidman et al., 1965). Loss of a functional β_4 subunit can impact the function of α_{1A} – containing VGCCs (P/Q-type).

The *tg* mouse mutation is an example of neuronal plasticity. These mice have a point mutation which causes an amino acid substitution of proline-to-leucine in the S5-S4 linker region of repeat domain II of the α_{1A} subunit (Fig. 4.1) (Fletcher et al., 1996). This leads to a non-functional α_{1A} subunit. These mice, therefore, control ACh release at the motor nerve terminal by N- and R-type VGCCs (Pardo et al., 2006). Despite showing an obvious phenotype, *lh* mice live to become adults. Consequently, I hypothesized that the *lh* mice might also be presenting some form of neuronal plasticity.

First I wanted to determine if and how ACh release at motor nerve terminals was affected by the alteration of the normal complement of VGCCs β subunit. To answer this question, intracellular recordings at the neuromuscular junction (NMJ) of adult *lh* and *wt* mice were obtained. The recordings were done in the presence of different concentrations of Ca^{2+} in physiological saline to determine if varying the Ca^{2+} concentration affected

Figure 4.1: Stucture of the α_{1A} subunit showing the *tg* mutation.

Schematic representation of the predicted transmembrane topology of the α_1 subunit, with the localization of the β-interaction domain (BID) marked in red. The cylinders indicate transmamebrane repeats (I-IV) of 6 transmembrane segments (1-6). The position of the *tg* mutation is indicated by an arrow (Adapted from Fletcher et al., 1996).



the frequency of spontaneous release. Also, an increase in Ca^{2+} concentration, leads to an increase in vesicle release, which in turn increases recycling. Therefore if this mutation affected the frequency of release, it would be easier to visualize by using a higher extracellular Ca^{2+} concentration, which would induce a faster frequency of release. There was no difference in amplitude or frequency of spontaneous release (MEPPs) in *lh* mice as compared to *wt* (Fig. 2.1).

This led to the question of what would happen if the charge carrier was changed to either Sr^{2+} or Ba^{2+}. These cations can substitute for Ca^{2+} in the generation of MEPPs (Anwyl et al., 1982; Elmqvist and Feldman, 1965; Mellow et al, 1982; Silinsky, 1978). Neurotransmitter release occurs through vesicle fusion which involves different Ca^{2+}-binding sites. These sites involved in asynchronous release are sensitive to Sr^{2+} and Ba^{2+}, while those involved in synchronous exocytosis are only sensitive to Sr^{2+} (Van Der Kloot and Molgó, 1994; Zefirov and Grigor'ev, 2010; Zefirov and Mukhamed'yarov, 2004).

Therefore, we substituted Ca^{2+} for Ba^{2+} or Sr^{2+} to determine if the asynchronous release process responded differentially to these ions (Fig. 2.2). There was no change in MEPP amplitude in *lh* as compared to *wt* (Fig. 2.2a). This implies that there is no difference in the amount of ACh each synaptic vesicle carries. However, when the frequency of spontaneous release was measured in the presence of Ba^{2+} or Sr^{2+}, *wt* mice showed an increase in frequency with the use of these cations. In *lh* mice, however, these

cations did not alter the frequency of MEPPs (Fig 2.2b).This could imply that this mutation has a presynaptic effect.

Nerve-evoked release of ACh (EPPs) and quantal content in *lh* mice in the presence of Ca^{2+} as a charge carrier, were significantly altered (Figs. 2.3 and 2.4). As stated before, Sr^{2+}, but not Ba^{2+}, can replace Ca^{2+} in supporting synaptic transmission, since the calcium-binding site for synchronous exocytosis is sensitive to Sr^{2+} ions (Dodge et al., 1969; Meiri and Rahamimoff, 1971; Miledi, 1966; Silinsky, 1977, 1981, 1985; Zefirov and Grigor'ev, 2010).

When utilizing Sr^{2+} as a charge carrier, I also observed that EPP amplitude and quantal content in *lh* mice were significantly decreased as compared to *wt* mice (Figs. 2.5 and 2.6). This may be due to the lower amount of current carried by Sr^{2+} in the VGCC.

Burgess et al. (1999) found that despite the loss of β_4 subunit, whole cell P-type current was present at normal amplitude in *lh* cerebellar Purkinje neurons. McEnery et al. (1998) showed that in forebrain and cerebellum of *lh* mice they express an immature form of N-type VGCCs, which in turn alters Ca^{2+} currents. The results presented so far in my dissertation opened the possibility that the electrophysiological changes I was measuring in *lh* mice could be due to alterations in the Ca^{2+} current flowing through VGCCs. To test whether this hypothesis is true, it would be interesting to measure Ca^{2+} current flowing through motor nerve terminals of *lh* mice and compared them to *wt* mice. These measurements could be performed in *triangularis sterni* (TS) muscle with its

intercostal nerves in adult *lh* and *wt* mice. Changes in the voltage potential arise due to the summation of currents flowing longitudinally outside of the axon and within the perineural sheath of motor neurons isolated from the intercostal nerve present in TS muscle. K^+ channel blockers {such as Tetraethylammonium (TEA) and 3,4-diaminopyridine (DAP)} should be used to unmask the Ca^{2+} currents present (that are normally obscured by the presence of K^+ currents) (Brigant and Mallart, 1982; Mallart, 1985; McArdle et al., 1981; Smith et al., 1995; Xu and Atchison, 1996). Since the β subunit is involved in modifying the kinetics of the VGCCs. I suspect that *lh* mice will have a decreased peak Ca^{2+} current flowing through them.

Based on these results, I also wanted to examine whether the vesicular release dynamics were affected by the absence of the β_4 subunit in *lh* mice, which could explain the decrease in ACh release. In order to do this, I performed FM1-43 staining of the nerve terminal. These experiments are designed to investigate how the *lh* mutation affects the availability of quanta release, the exocytosis of vesicles and their recycling (Betz and Bewick, 1993; Reid et al., 1999). The entire process of destaining took approximately 3.5 min to be completed in *wt* mice; however at that time there was still a large amount of fluorescence remaining in nerve terminals of *lh* mice (Fig. 2.7). It took ~ 15 min for *lh* preparations to achieve a level of destaining similar to that measured in *wt* mice (Fig. 2.8). Thus the vesicle release process is significantly slower in *lh* mice. However this finding opens additional questions. The slower process could be due to a releasable pool size problem or a reduced Ca^{2+} current. To test this, vesicle release was induced by two

different mechanisms: α-LTx (Hubbard et al., 1968; Rosenmund and Stevens, 1996; Rossetto et al., 2004; Sons and Plomp, 2006; Tedesco et al., 2009; Xu et al., 2002) or hypertonic solution (Palma et al., 2011; Sons and Plomp, 2006). In each case, the rate of the destaining process was the same in *lh* and *wt* mice; suggesting that a decreased Ca^{2+} current might contribute to the slow process in the release, and not the vesicle pool size (readily releasable or reserve pool) in *lh* mice. Therefore, destaining of FM1-43 appeared to be slower and less complete in *lh* mice. However, it would be of interest to determine if the vesicle release process is altered between *lh* and *wt* mice, when Ca^{2+} is substituted for Sr^{2+} or Ba^{2+} when performing FM1-43 experiments.

In conclusion, ACh release at somatic motor nerve terminals is significantly altered in the *lh* mice. This might be due to an effect in the Ca^{2+} current which could then translate to a slower vesicle release or recycling process, leading to a decrease in ACh release. This could explain the abnormal phenotype seen in *lh* mice.

In the future it would be interesting to study what would happen if these animals were treated with DAP. DAP blocks K^{+} channel efflux in nerve terminals. This way the duration of action potentials is increased, allowing VGCCs to remain open for longer periods of time. Hence, a greater amount of ACh is released from the motor nerve terminal. According to the results of this dissertation, the *lh* mutation affects ACh release, which could account for their phenotypic "*lethargic*" behavior, but it would be interesting to know what would happen if we increase the availability of ACh by inhibiting motor nerve terminals K^{+} channels. Would this change the phenotype of these

mice? Would it alter their behavior since they would have more ACh available to stimulate the muscle? This experiment could be done in two ways. In the first part diaphragm dissections could be treated with an inhibitor of DAP and then intracellular recordings from the NMJ could be performed; this would enable one to see if there was a change in the postsynaptic response. If there is an increase in postsynaptic response as measured by an increase in EPP amplitude which translates to an increase in quantal content, then I could potentially go to animal trial. Mice would be treated with an DAP and the progress of the mice would be assessed by behavioral tests such as foot print test to quantify motor function and the stride length, rearing-climbing to assess motor movement and coordination, and grip strength test to measure forelimb grip strength as an indicator of neuromuscular function. Additionally weight changes and eating habits should be monitored, since *lh* mice have reduced body weight as compared to *wt* mice. Since DAP has been tried in LEMS patients and proven to have negligible side effects (perioral and digital paresthesia), and laboratory studies showed no evidence of toxicity affecting liver, renal, hematologic, endocrinologic, encephalographic, or electrocardiologic function (Sanders et al., 2000), therefore, it could be safe to consider this treatment for *lh* mice, although constant monitoring of the animals is advised.

Based on the fact that *lh* mice have a decrease in ACh release and a slower process of release, I wondered if there was an alteration in the Ca^{2+} current, then, what was responsible for this alteration. Therefore I wanted to determine if the P/Q-type VGCCs are involved in controlling ACh release at adult *lh* motor nerve terminals. Additionally, I also wanted to assess the possible involvement of other types of VGCCs in controlling ACh release. Finally, I wanted to determine which β subunits were

substituting for the absence of β_4. The diaphragm muscle presents a scarcity of VGCC protein levels. When performing western blots in this tissue, the western blot was not sufficiently sensitive to detect VGCCs subunits in the presynaptic area. Therefore, I performed western blots to determine the protein levels in cerebellum of *lh* and *wt* mice. Since *lh* animals lack the β_4 subunit, I first wanted to determine whether this mutation affected the protein levels of the remaining different β subunits in cerebellum of adult *lh* mice. Additionally I wanted to assay for α_{1A} levels since the β_4 subunit normally co-associates with α_{1A}; therefore I wanted to test if the absence of β_4 affected the levels of α_{1A}. Protein level analysis showed that even though *lh* animals lack the β_4 subunit, the protein levels of the α_{1A} subunit are the same for *lh* and *wt* animals. This could be due to the significantly higher levels of β_1 and β_3, which might compensate for the lack of β_4 (Fig. 3.1).

This could imply that ACh release was controlled by P/Q-type VGCCs, in which the α_{1A} subunit was abnormally associating with either β_1 and/or β_3 subunit. To determine if this was correct, I performed intracellular recordings using ω-agatoxin IVA (ω-Aga IVA) (a specific antagonist of P/Q-type VGCCs). ω-Aga IVA significantly decreased evoked ACh release in *lh* and *wt* animals (60% and 76%, respectively) (Fig. 3.2). Since the effect of ω-Aga IVA was lower in *lh* mice, this could be explained by the fact that maybe other types of VGCCs are also controlling ACh release in adult *lh* mice. To determine if this was the case, I performed additional intracellular recordings in the

presence of other VGCCs antagonists. The use of specific antagonists for L- and N-type VGCC (nimodipine and ω-Ctx GVIA respectively) did not affect quantal content, implying that neither L- nor N-type VGCCs control ACh release in either *wt* or *lh* mice. However, SNX 482 an R-type VGCC antagonist significantly decreased quantal content in *lh* (46%), but not *wt* mice (8%) (Fig. 3.2), thus implying that R-type VGCC contribute to the control of the ACh release at motor nerve terminals of adult *lh* but not *wt* mice.

Co-application of ω-Aga IVA and SNX 482 significantly reduced ACh release in *lh* mice to a level similar to that observed after the application of ω-Aga IVA alone in *wt* mice. This suggests that ACh release in *lh* animals is controlled by P/Q- and R- type VGCCs, and that both types seem to contribute to a similar extent (Fig. 3.3).

The western blots showed increased level of β_1 and β_3 subunits, which might be compensating for the absence of β_4 subunit. Additionally, electrophysiology had shown that ACh release is controlled by P/Q- and R-type VGCCs in *lh* mice. I, therefore, wanted to verify the presence of the different α_1 and β subunits at the NMJ of adult mice by performing immunohistochemistry in sections of EDL muscle. I chose this muscle to permit me use the diaphragm for electrophysiological studies and the EDL for immunohistochemical studies from the same animals. Some subtle differences exist between diaphragm and EDL. The diaphragm is a mix fiber type of muscle, and the EDL is a fast twitching type of muscle. However, there is enough similarity between them that it will not present a problem. EDL sections were stained with specific antibodies against the various α_1 and β subunits as well as the nicotinic ACh receptor (nACh - as a postsynaptic marker).

When the relative amount of fluorescence was quantified for each subunit in both genotypes (Fig 3.5), I found a significant increase in α_{1E}, β_3 and β_1 in *lh* mice as compared to their *wt* littermates. However, the levels of α_{1A} in *lh* mice were not significantly different from *wt* mice. These results are consistent with my western blot and electrophysiology findings. When the juxtaposition of the VGCCs subunits against the somatic nACh receptor was quantified, it showed that in *lh* mice there is a 58% and 44% juxtaposition of α_{1A} and α_{1E} respectively, while in *wt* the α_{1A} subunit had a 73% juxtaposition. With regards to the β subunits, in *lh* mice β_1 and β_3 juxtaposed with the nACh at 88% and 65%, respectively (Fig. 3.6).

The immunohistochemistry data confirmed the electrophysiology data; both α_{1A} and α_{1E} staining overlap that of nicotinic receptors at the NMJ. Determining additionally that there is an increase in β_3 and β_1 subunit in *lh* mice, which might compensate for the absence of β_4 subunit. Taken together, these results suggest that ACh release is mediated by P/Q- as well as R-type VGCCs in *lh* mice. However, it would be interesting to confirm these results by determining whether there is a change in the mRNA level of the different β subunits. RT-PCR assays could be performed, in which mRNA is isolated from spinal cords of *wt* and *lh* mice.

We know that the β_3 subunit is extensively expressed in the brain, especially in the hippocampus (Ludwig et al., 1997; Namkung et al., 1998). It has been shown that the $\beta_3^{-/-}$ mouse exhibits enhanced long term memory and N-methyl-D-aspartate receptor

(NMDAR)-dependent long term potentiation (LTP) as well as NMDAR-mediated synaptic responses and increase NR2B level in the hippocampus (Jeon et al., 2008). It is therefore possible that β_3 subunits normally suppress NMDA receptors, although whether this effect is a result of a direct interaction remains to be determined (Jeon et al., 2008). Based on these results, and considering that the β_3 subunit is significantly increased in *lh* mice, it would be interesting to determine whether these animals have an impairment in memory formation. To study memory formation I propose two separate sets of tests. First a set of behavioral tests, given the low motor coordination these animals present, some commonly used tests to evaluate memory formation are not going to be able to be used (such as the case of water maze or contextual fear conditioning). Therefore, I propose we perform social transmission of food preference and novel object recognition memory task. Second, I propose we evaluate LTP by whole-cell patch clamp recording on hippocampal slices and compare the results to those obtained in the *wt* littermates.

Additionally, Lambert-Eaton Myasthenic Syndrome (LEMS) is an autoimmune disease, characterized by muscle weakness, in which autoantibodies primarily target the α_{1A} subunit of the P/Q-type calcium channels (Flink and Atchison, 2003; Hewett and Atchison, 1992; Nagel et al., 1988; Suzuki, 2010; Takamori, 2008). One goal of future studies would be to determine how *lh* mice respond to LEMS antibodies. Since *lh* mice have both P/Q- and R-type VGCCs we could test whether R-type VGCCs are also targeted by LEMS autoantibodies as are P/Q-type. Additionally we could test whether there is a compensation by different VGCCs localized at the motor nerve terminals, since it has been shown that after treating *wt* mice with LEMS plasma they compensate with L-type calcium channels for the attack on the P/Q-type performed by LEMS autoantibodies

(Flink and Atchison, 2002). In order to perform this study, *lh* mice would be exposed to LEMS plasma by passive transfer of 1.5 ml of plasma from LEMS patients over a period of 30 days (Hewett and Atchison, 1992; Smith et al., 1995), after which time the mice would be sacrificed and evaluated to determine whether LEMS antibodies had any effect on them. The involvement of the different VGCCs would be assessed by intracellular recordings of the NMJ in the presence of different VGCC antagonists. The phrenic nerve will be stimulated at 0.5 and 50 Hz to determine whether there is facilitation, a characteristic of LEMS, which would show whether the passive transfer was successful and if the mice acquired LEMS-like characteristics. Additionally, immunohistochemistry could be performed to determine the localization of the different subunits of VGCC present in animals treated with LEMS plasma.

Although some of my results are similar to those presented by Kaja (2007), there are several areas of disagreement. When evaluating which VGCCs control ACh release, he used only one antagonist, ω-Aga IVA which is the specific antagonist for the P/Q-type VGCCs, and did not investigate the possible involvement of other VGCCs. Moreover he did not evaluate protein levels or subunits localizations by the use of western blots or immunohistochemistry studies to confirm the results he obtained. Additionally, he determines that P/Q-type VGCCs are the only ones responsible for the control ACh release in *lh* mice. These differences between our results could be explained by the age difference between the animals we used. While he worked with 6 wks old mice, I performed all the experiments present in this dissertation using mice between the ages of 3 to 9 mos. Several studies have demonstrated developmental changes in the expression of the α_1 subunits of VGCCs (Gray et al., 1992; Rosato Siri and Uchitel,

1999). It has been determined that β subunits regulate the channel properties and targeting of α_1. Studies done in rat brain showed that the immature N-type VGCC is comprised mainly of β_{1b}, while the mature N-type is comprised of $\beta_3 > \beta_{1b} > \beta_4$ (Ludwig et al., 1997; McEnery et al., 1998; Tanaka et al., 1995; Vance et al, 1998). It is not clear what is the developmental pattern of expression for the β subunits in nerve terminals, and if the pattern of expression is finalized by 6 wks, especially in *lh* mice. Full expression of α_{1E} and the different β subunits may not occur until later than 6 wks postnatal in *lh* mice. Thus at the comparatively young age of mice in which Kaja (2007) examined ACh release, there might have been a greater dependence on P/Q-type, as seen by a larger percentage of sensitivity of his animals to ω-Aga IVA, and a lesser contribution of R-type channels than in 3 to 9 mo old animals that I used. These differences could possibly lead to differences in our results.

Kaja's work showed that the *lh* mutation did not affect MEPP amplitude or frequency, or in EPP amplitude or quantal content. Something that over the length of this dissertation I have shown using different techniques to assess for ACh release (both spontaneous and nerve evoked release). The study performed by Kaja et al. (2007) did not include the use of different divalent cations to substitute Ca^{2+} as charge carriers, which might explain why he did not see any difference in MEPP frequency, since there is no difference in the frequency of spontaneous release when using Ca^{2+} as a charge carrier; but these differences become apparent when substituting Ca^{2+} by Ba^{2+} or Sr^{2+} (chapter 2). Another possible explanation for our differences might be methodological.

While I performed recordings from 5 to 10 end-plates of up to 5 minutes each; Kaja's work was done by recording at least 30 EPPs and 30 MEPPs at each NMJ. At their reported frequencies in their article (0.81 MEPPs/s in *lh* and 0.93 MEPPs/s in *wt*) this implies recordings no longer than 40 s. I sampled over a much longer interval. At a MEPP frequency of 1Hz for 5 minutes, I sampled ~300 MEPPs. Given the fact that we both stimulated EPPs at 0.5 Hz, this implies his recordings were about 90 s long. At a stimulation of 0.5 Hz, for 5 minutes I sampled 150 EPPs. Short time intervals could reflect inaccurate results, which could not be representative of what is going on over longer intervals. This would need to be examined more rigorously, by repeating the experiments using his protocol.

This is not the first time that neuronal plasticity at the NMJ has been reported (Flink and Atchison, 2002; Pardo et al., 2006; Xu et al., 1998). The *lh* mutation seems to present a compensatory mechanism in which VGCCs present at motor nerve terminals are formed by α_{1A}-β_1/-β_3 and α_{1E}-β_1/-β_3 (Fig. 4.2). This is consistent with the fact that the AID consensus sequence is highly conserved among all the subunits (de Waard et al., 1994). Additionally, the work done in rabbit brain membranes by Liu et al. (1996), has shown that there is a preferential α_1- β pairing ($\beta_4 > \beta_3 >> \beta_{1b} \geq \beta_2$). Although my findings do not show a preference between β_3 and β_1 pairing with either α_{1A} or α_{1E}. This difference might suggest that the interaction of the α_{1A} and β subunits *in vivo* may be dependent upon the species, spatial and temporal expression of β subunits and the affinity of α_{1A}-β subunit interaction. However, this abnormal association between α_{1A}- β_1/-β_3

and the presence of α_{1E} might be a possible cause for the slower vesicle release process observed in *lh* mice.

Figure 4.2: Proposed model of the NMJ of adult *lh* mice.

(a) In motor nerve terminals of adult *wt* mice we find α_{1A} which normally co-associates with β_4 since in *wt* ACh release is primarily controlled by P/Q-type VGCCs. Additionally *wt* mice have a faster vesicle release process. (b) In motor nerve terminals of adult *lh* mice there is localization of α_{1A} and α_{1E} which seem to associate with either β_1 or β_3 indiscriminately. Thus in *lh* mice ACh release is controlled by P/Q- and R-type calcium channels, which are also characterized by a slower release process.

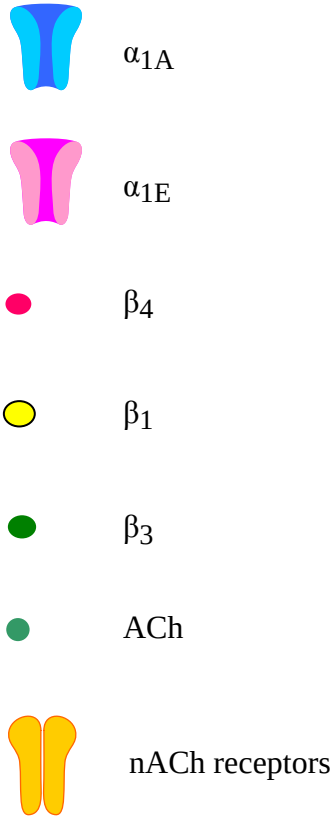


Figure 4.2 (cont'd)

(a) *wt* NMJ

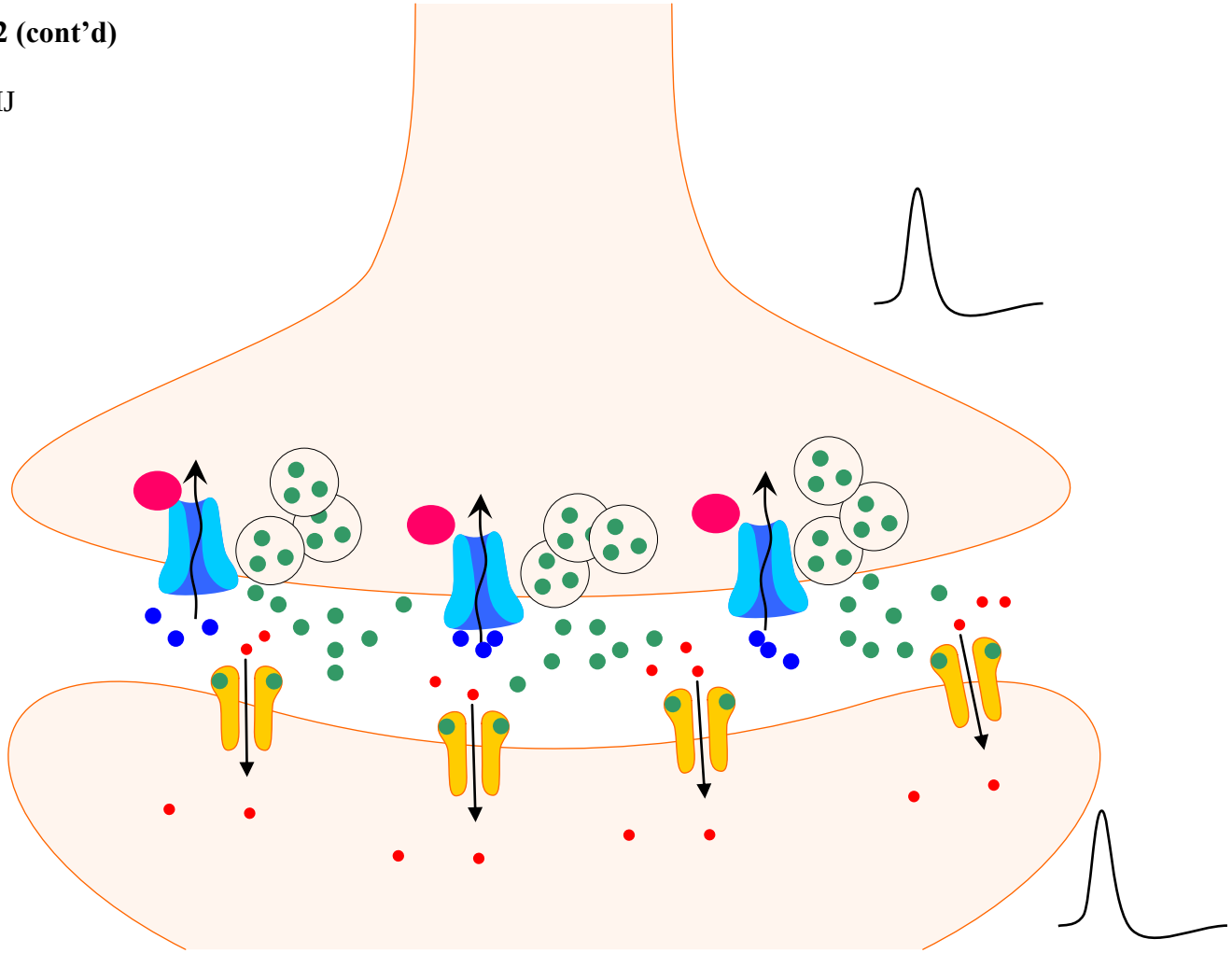
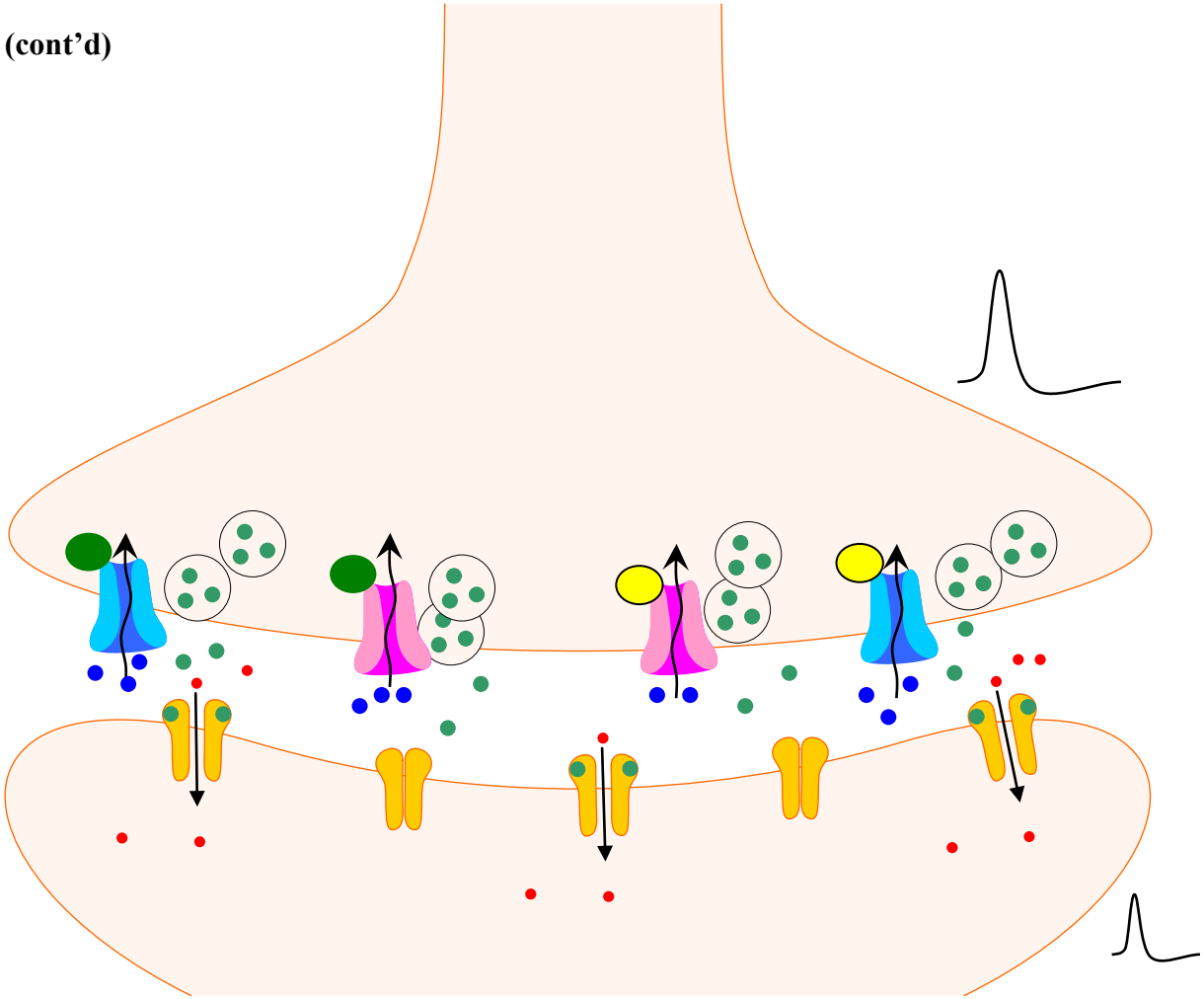


Figure 4.2 (cont'd)

(b) *lh* NMJ



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