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SNAP-25 is a component of a ubiquitous SNARE complex required for evoked neuroexocytosis in GABAergic neurons

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SNAP-25 IS A COMPONENT OF A UBIQUITOUS SNARE COMPLEX REQUIRED FOR EVOKED NEUROEXOCYTOSIS IN GABAERGIC NEURONS

BY

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B.S., Biology, University of New Mexico, 2001

DISSERTATION

Submitted in Partial Fulfillment of the
Requirements for the Degree of

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Dedication

I dedicate this dissertation to my beautiful wife Charlene and my two precious daughters, Isabella and Gabriella. It is through their unconditional support, undying patience, and infinite understanding that I was able to reach this important milestone. I cannot begin to repay them for the numerous sacrifices they gracefully made over the years.

Individually, I would like to thank my oldest daughter, Isabella, for her inquisitive nature, serving as a continual reminder that knowledge belongs to those who seek it and, at any age, one should never stop questioning the world around us. For Gracie, I owe my gratitude for the moments of levity she provides, which extinguish panic, neutralize anxiety, and put into perspective the true priorities in life. Lastly, I pay tribute to my wife, who single-handedly has galvanized my success as a father, husband, physician, researcher, and a man.

To have such special people in my life is truly a blessing and I cannot begin to enumerate the many contributions they have made. However, I can say that it is with seemingly uncanny perception and impeccable timing that they supply the hugs, cheers, and kisses that not only help me maintain my successes, but also propel me to a higher level in search of new goals. Perhaps more importantly, my family gives the encouragement that, during times of seemingly crushing failure, replenishes my spirit and reinforces my resolve. They are everything I will ever need in this life, and their unconditional love and

unrestrained laughter will continue to eliminate my notions of impossible and perpetually redefine my potential.

For my Dissertation Committee, I will never be able to express the gratitude I have for their invaluable guidance and support during this journey, especially during times when my mentor was physically unable to fufill his role due to unfortunate medical circumstances. Word by word I composed this dissertation, but without the guidance of my committee members, the quality of the following manuscript would have never truly reflected the heart, soul, and determination I poured into the project over my five years of training. For that reason, I wholeheartedly thank Donald Partridge, Lee Anna Cunningham, Dusanka Deretic, and Fernando Valenzuela.

Lastly, for my mentor, friend, and brother, Michael C. Wilson, there will never be enough words or enough time to express the impact of your influence in my life. Sir, I humbly thank you for not only allowing me the priviledge to work in your laboratory, but also for caring enough to challenge me at every turn, demanding performance at the capacity I had yet to discover, but one you knew I possessed all along. With every publication I write, surgery I perform, and patient I treat, there will be the knowledge that my skills and professionalism began as a seed that germinated in the Wilson Labs. You have changed my life in profound ways and as I carry your teachings forward, you will continue to change the lives of many.

SNAP-25 IS A COMPONENT OF A UBIQUITOUS SNARE COMPLEX REQUIRED FOR EVOKED NEUROEXOCYTOSIS IN GABAERGIC NEURONS

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Abstract

The processes of vesicular trafficking and membrane fusion are fundamental to nervous system development and communication among neurons within integrated circuits. The regulated release of several neurotransmitters is dependent on synaptosomal-associated protein 25 kDa (SNAP-25)-containing soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) core complexes. The requirement for this fusogenic machinery in evoked neurosecretion has been repeatedly demonstrated through the use of mutant mouse models and neurotoxin blockades. However, the existence of a functional role for a SNAP-25-containing SNARE complex has not been shown in the major inhibitory neurotransmitter system of the brain, gamma-aminobutyric acid (GABA). To determine whether SNAP-25 participates in the evoked GABAergic neurotransmission, we investigated the expression and function of this protein in inhibitory terminals. In addition, we identified the major SNAP-25 isoform expressed by mature GABAergic neurons. The results presented here provide compelling evidence that SNAP-25 is critical for evoked GABA release and is expressed in the presynaptic terminals of mature GABAergic neurons, consistent with its function as a component of a fundamental core SNARE complex required for stimulus-driven neurotransmission. Furthermore, we conclude that SNAP-25b is the predominant isoform expressed in central inhibitory neurons of the adult brain.

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1. Introduction

Intracellular trafficking and membrane fusion are important processes for all cells throughout the body. However, in neurons, these events are tailored to facilitate high-speed signal propagation and regulated secretion. It is the calcium triggered release of neurotransmitter from the presynaptic terminal, via vesicle fusion, that allows neurons to communicate with other neurons as well as other tissue targets. In the nervous system, stimulus induced fusion events are carried out by the intricate synchronization of numerous protein-protein interactions. Specifically, the basic neuroexocytotic machinery utilized for action potential-dependent transmission is comprised of the neural soluble NSF attachment receptor (SNARE) proteins, synaptosome-associated protein of 25 kDa (SNAP-25), vesicle associated membrane protein 2 (VAMP-2, also known as synaptobrevin), and syntaxin 1a. These three specialized proteins interact and assemble into a SNARE complex that is the core component required for dynamic signaling in many neurotransmitter systems.

Neurons are highly polarized cells and neurosecretion, via the docking and subsequent fusion of synaptic vesicles, occurs at restricted, morphologically defined domains along the plasma membrane of the presynaptic terminal. During neuronal stimulation, vesicular fusion occurs by Ca^{2+}-dependent exocytosis, causing neurotransmitter release into the synaptic cleft, followed by endocytotic retrieval of the vesicle membrane components. Finally, secreted neurotransmitter activates receptors on the post-synaptic cell, resulting in the

relay of messages along a neural pathway, completing the underlying signaling process of complex higher order functions that define the characteristics and capabilities of specialized brain networks.

1.1 The Discovery of Components within the Neural SNARE Complex

A major insight to the process of neurosecretion was the observation that the neural SNAREs are members of a larger, highly conserved superfamily of proteins that are involved in a wide variety of vesicle trafficking events in the eukaryotic cell, such as the generation or budding of new vesicles, targeting of the vesicle to a prospective release site on the plasma membrane, and finally, mediating the subsequent membrane fusion necessary for secretion of vesicular cargo (for reviews, see Bonifacino and Glick, 2004; Hong, 2005). Along with specialized functions such as the regulated secretion of neurotransmitters, SNARE proteins are also responsible for constitutive vesicular trafficking events, such as those needed to localize membrane-bound proteins to the surface of the cell. This vesicular trafficking is a fundamental mechanism for cellular homeostasis as cargo encapsulated in vesicles is transported from a donor to a targeted acceptor compartment where membrane fusion occurs, creating a directed flow of proteins, lipids, and secretory factors throughout the cell. While membrane fusion has been studied for decades, research conducted over the last quarter century has elucidated the molecular components involved in the

specialized vesicular trafficking and membrane fusion that occurs within many diverse and specialized tissue systems, including the brain.

Early investigations of the yeast species Saccharomyces cerevisiae provided the first identification of proteins involved in vesicle trafficking: Sec17, Sec18, Sec20 and Sec22 (Novick et al., 1980; Eakle et al., 1988). Shortly afterward, landmark studies began to describe mammalian homologues of these yeast proteins, such as the N-ethylmaleimide-sensitive fusion protein (NSF) and soluble NSF-attachment proteins (SNAPs), providing the framework necessary to dissect components of the exocytotic machinery (Wilson et al., 1989; Clary et al., 1990). Early studies identified VAMP-2, syntaxin, and SNAP-25 with expression exclusively in neurons, particularly in brain regions with high concentrations of presynaptic terminals, known as neuropil (Trimble et al., 1988; Baumert et al., 1989; Oyler et al., 1989; Bennett et al., 1992; Inoue et al., 1992). Examination of the subcellular localization of these proteins revealed that they were predominately segregated and associated to membranes of the presynaptic terminal and synaptic vesicles. In addition to evidence that SNAP-25, VAMP-2, and syntaxin 1a were homologues to yeast proteins involved in vesicular trafficking, it was the strong interaction of these three proteins with two required cofactors for membrane fusion, NSF and SNAPs, that suggested their involvement in the basic neuroexocytotic machinery and led to the terminology of soluble N-ethylmaleimide sensitive factor attachment protein receptors or

"SNAREs" (Wilson et al., 1989; Clary et al., 1990; Aalto et al., 1993; Sollner et al., 1993; Brennwald et al., 1994). The SNARE hypothesis of neurotransmission was strengthened as further studies confirmed that the mechanism of action for the most potent blockers of neurotransmission, botulinum (BoNT) and tetanus (TeNT) neurotoxins, was the specific targeting and cleavage of VAMP-2, SNAP-25, and syntaxin 1a, demonstrating the specialized function of these SNARE proteins in neurotransmitter release (Schiavo et al., 1992; Blasi et al., 1993; Blasi et al., 1993). Later, ultrastructural analysis established that VAMP-2, SNAP-25, and syntaxin 1a formed a heterotrimeric, four-barreled coiled-coil structure, designated as the neural SNARE complex, that comprised the minimal or core machinery necessary for vesicular fusion (Sutton et al., 1998; Weber et al., 1998).

1.2 Structural properties of neural SNARE proteins

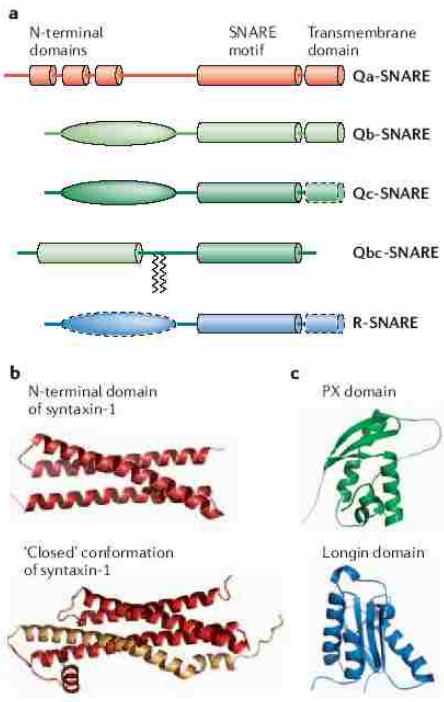
Just as the neural SNARE complex mediates regulated neuroexocytosis, other SNARE complexes exist and are individually specialized to facilitate virtually every element of intracellular transport, including vesicular trafficking as well as constitutive and stimulus-driven delivery of plasma membrane proteins (reviewed in Jahn et al., 2003; Hong, 2005). SNARE proteins are classified by an alpha-helical motif which is comprised of a heptad repeating sequence that spans 60-70 residues that have been evolutionarily conserved in eukaryotes, from yeast to humans (Terrian and White, 1997; Weimbs et al., 1997; Weimbs et al., 1998;

Bock et al., 2001; Fasshauer, 2003). However, aside from the common motif that defines the proteins within this group, structural organization of the C- and N-terminals of SNAREs can vary widely.

Carboxyl terminal structure in SNARE proteins

Carboxyl terminal structure beyond the SNARE motif differs among SNARES, but most commonly consists of a short linker region that is followed by a single transmembrane domain (Fig. 1.1A), such as those found in the neural SNAREs syntaxin 1a and VAMP-2 (reviewed in Fasshauer, 2003; Hong, 2005). The transmembrane domain may serve several functions in SNARE proteins aside from allowing their insertion into the plasma membrane, including driving SNARE complex assembly, providing a site for accessory protein binding, and initiating fusion pore formation during exocytosis (Poirier et al., 1998; Margittai et al., 1999; Lewis et al., 2001; Han et al., 2004; Xu et al., 2005). In contrast, some SNARE proteins, such as SNAP-25, lack a transmembrane domain, but can

Figure 1.1 Stuctural motifs in common SNARE proteins



A series of schematic drawings illustrating various conserved and divergent motifs of SNARE proteins. Reproduced from Jahn and Scheller, 2006.

undergo post-translational modifications, specifically through the addition of hydrophobic molecules to targeted residues, in order to interact with or anchor themselves to the plasma membrane. Farnesylation and palmitoylation are two such mechanisms by which a 15-carbon farnesyl or palmitate fatty acyl chain is covalently linked, via a thioester bond, to cysteine residues encoded within SNARE proteins such as SNAP-23, Ykt6 in yeast, and the “cysteine quartet” (residues 84, 85, 90, and 92) of SNAP-25 (Hess et al., 1992; Veit et al., 1996; McNew et al., 1997; Vogel and Roche, 1999). Interestingly, those SNAREs that contain a transmembrane domain, such as VAMP-2, can also be palmitoylated, which has been shown to increase their half-life by preventing ubiquination and subsequent degradation (Couve et al., 1995; Veit et al., 2000; Valdez-Taubas and Pelham, 2005).

Amino terminal structure in SNARE proteins

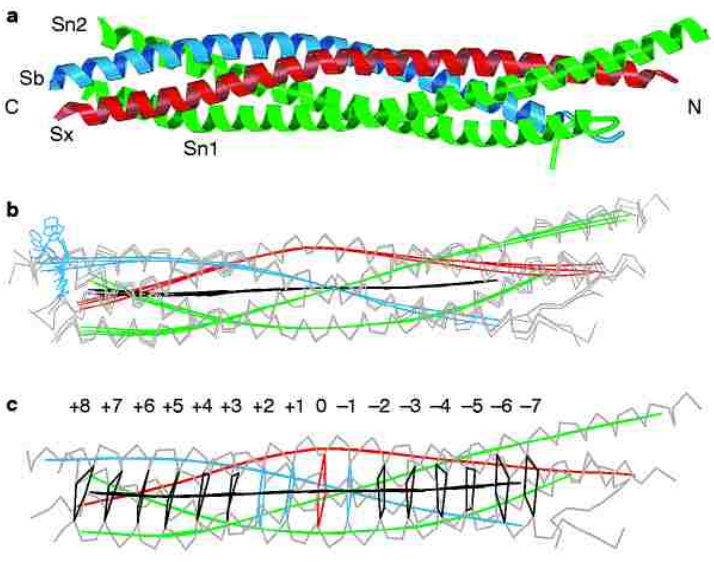
SNARE proteins also display considerable diversity in amino terminal structure, which can range from little or no secondary structure to complex folding domains (Misura et al., 2002; Dietrich et al., 2003). For example, SNAREs associated with vesicular membranes are divided into two classes, “longins” (Fig. 1.1C), which contain a structured N-terminal formation, and “brevins,” such as the neural SNARE VAMP-2, that possess only a few amino acids beyond the SNARE motif structure (Filippini et al., 2001; Rossi et al., 2004; Rossi et al., 2004). The N-terminal domain of syntaxin 1a consists of an elaborate antiparallel

bundle of three alpha helices, termed Habc (Fig. 1.1B), which form autonomously at the end of a flexible linker region (Fernandez et al., 1998; Lerman et al., 2000; Margittai et al., 2003). When syntaxin 1a is in a monomeric form, the Habc domain plays an important regulatory role causing this structure to fold back and self-associate with the SNARE motif to form a closed conformation that shields the protein, and thereby regulating complex assembly and possibly ensuring that binding occurs only with specific molecular targets (Kee et al., 1995; Fernandez et al., 1998; Margittai et al., 2003). However, the more significant contribution of N-terminal modifications is the ability to act as a site of interaction and recruitment for accessory proteins to the SNARE complex necessary to complete the ensemble of proteins and cofactors that are tailored to a specific type of exocytosis. For example, sec1/munc18-related (SM) proteins, essential components of the exocytotic machinery, interact with individual SNAREs, such as munc 18-1 in binding to syntaxin 1a, as well as facilitate the assembly and stabilization of the heterotrimeric neural SNARE complex through specific binding to N-terminal domains (Hata et al., 1993; Garcia et al., 1994; Pevsner et al., 1994; Dulubova et al., 1999; Toonen and Verhage, 2003). In addition, N-terminal interactions between the neural SNAREs are important for progression through the core complex assembly pathway as well as for the mechanisms responsible for priming vesicles and driving membrane fusion (Zhong et al., 1997; Fasshauer and Margittai, 2004; Borisovska et al., 2005; Pobbati et al., 2006).

1.3 Neural SNARE complex assembly and structure

The minimal or core requirement for synaptic vesicle fusion consists of a heterotrimeric SNARE complex comprised of SNAP-25 and two single-pass transmembrane proteins: syntaxin 1a and VAMP-2 (Weber et al., 1998). The fully assembled core complex has a characteristic formation of four parallel coiled-coil amphipathic alpha helices, one each from syntaxin 1a and VAMP-2 and two from SNAP-25, with contains two SNARE motifs separated by a flexible linker region (Chapman et al., 1994; Sutton et al., 1998). As individual SNARE regions combine to form the four barreled helix, non-polar residues from each alpha helix are aligned and interact to create the internal hydrophobic core of the neural SNARE complex (Fig. 1.2A).

Figure 1.2 The Neural SNARE complex structure



Four-barreled neural SNARE structure. **A**. assembly of four SNARE motifs, one from syntaxin 1a (blue), one from VAMP-2 (red), and two from SNAP-25 (green). **B,C**. Backbone structure showing location of hydrophobic layers. Reproduced from Sutton et al., 1998.

Originally, SNAREs were classified by their expression in a particular subcellular compartment, which was based on their localization to either the vesicular membrane (v-SNARE) or to the subsequent area of fusion, or target membrane (T-Snare Sollner et al., 1993).

The classification of t- or v-SNAREs is not the most appropriate for some SNARE proteins, which can be involved in many different vesicle trafficking pathways, causing their subcellular localization to vary, but in the specialized neural SNARE complex, a clear segregation of the two t-SNAREs, syntaxin 1a and SNAP-25, and the v-SNARE VAMP-2 is maintained (Trimble et al., 1988; Baumert et al., 1989; Oyler et al., 1989; Bennett et al., 1992; Grote et al., 1995; Walch-Solimena et al., 1995; Mandic and Lowe, 1999; Pennuto et al., 2003; Mitchell and Ryan, 2004; Bonanomi et al., 2007). This segregation is required for membrane fusion as SNARE proteins must be located opposite of each other on the two lipid bilayers destined to fuse (Nichols et al., 1997). To maintain this requirement, the neural SNAREs, as shown for syntaxin 1a, are actively sorted and returned to their respective membranes after fusion and subsequent vesicle retrieval (Mitchell and Ryan, 2004).

There are 15 distinctive sites of amino acid side chain interaction throughout the coiled-coil structure that represent the major grooves found in the core SNARE complex structure (Sutton et al., 1998). These grooves, or layers, are a series of hydrophobic interactions that traverse the four-barreled helix, save for one unique ionic interaction bisecting the complex that occurs between charged residues of glutamine or arginine conserved within the center of each SNARE motif (Fig 1.2B,C). The highly conserved expression of either a glutamine (Q) or arginine (R) at this position in the amphipathic helix is characteristic for each SNARE protein and has led to an alternative classification system used for the

SNARE family (Fasshauer et al., 1998). The strict expression of either a Q or R residue has functional implications in assembly as the four barreled neural SNARE complex, like all SNARE complexes, is comprised of one R-SNARE motif, contributed by the sole v-SNARE, VAMP-2, and three Q-SNARE regions termed Qa, which is supplied by syntaxin 1a, along with Qb and Qc, provided by SNAP-25 (Fasshauer et al., 1998; Sutton et al., 1998; Bock et al., 2001; Antonin et al., 2002).

Before complex assembly, monomeric SNAP-25 and synaptobrevin/VAMP-2 possess very little, if any, significant secondary structure (Cornille et al., 1995; Fasshauer et al., 1997; Fasshauer et al., 1997; Hazzard et al., 1999; Margittai et al., 2001). In contrast, syntaxin 1a, when isolated from cognate SNAREs, adopts complex secondary structure, especially at the N-terminal Habc domain, that is comprised of three alpha-helices which independently fold into a stable antiparallel bundle through hydrophobic and hydrophilic interactions (Fernandez et al., 1998; Lerman et al., 2000; Misura et al., 2000; Margittai et al., 2003). The Habc domain of syntaxin 1a monomers will then self-associate the H3 region of the Qa SNARE motif (Hanson et al., 1995; Dulubova et al., 1999; Misura et al., 2001), to form a four-helix structure to form a “closed” conformation stabilized by munc 18-1 that protects against non-specific interactions (Misura et al., 2000; Margittai et al., 2001). In addition, the C-terminal transdomain region of syntaxin 1a monomers can interact to form homodimers (Kroch and Fleming, 2006).

While complex formation is propelled by the spontaneous association of SNARE motifs, the assembly pathway is defined as a progression through several intermediate structures, suggesting several opportunities for regulation of fusion kinetics. Previous studies illustrated that the SNARE proteins Sso1p and Sec9p, yeast homologues for syntaxin 1a and SNAP-25, respectively, form a heterodimer that provides the necessary binding site for Sncp (synaptobrevin/VAMP-2 homologue Rice et al., 1997; Nicholson et al., 1998; Fiebig et al., 1999). An identical stage during the assembly of the neural core complex occurs, wherein a partially helical Qabc intermediate, consisting of the three Q-SNARE motifs derived from the N-terminal interaction of syntaxin 1a and SNAP-25, is formed prior to heterotrimertic SNARE complex formation, in a rate-limiting step where SNAP-25 displaces the SM protein munc 18-1 and interacts with the now exposed Qa SNARE motif (Zhong et al., 1997; Lang et al., 2002; An and Almers, 2004; Fasshauer and Margittai, 2004).

The Qabc intermediate, known as the acceptor complex, is stabilized by the temporary contribution of a fourth helix from a surrogate protein, which, in essence, acts act as a place-holder prior to VAMP-2 association. Several proteins possess portions of a SNARE motif (Scales et al., 2002; Echarri et al., 2004) and may interact with the Qabc acceptor complex, conferring the necessary stability. One such protein in particular, tomosyn, contains a pseudo R-SNARE sequence in its C-terminus and interacts weakly with Qabc acceptor complex until replaced by an R-SNARE, suggesting replacement of this

surrogate SNARE motif occurs as a cognate protein becomes available for finialized core complex assembly (Hatsuzawa et al., 2003; Pobbati et al., 2004; Sakisaka et al., 2004). Additionally, in the absence of VAMP-2, an additional syntaxin 1a molecule can interact with the acceptor complex to form a four-barreled binary intermediate complex with a stoichiometry of two syntaxin molecules to one SNAP-25 molecule, closely resembling the fully assembled tertiary SNARE complex (Fasshauer et al., 1997; Margittai et al., 2001). However, because this 1:2 SNAP-25:syntaxin 1a intermediate structure is less stable than the heterotrimeric neural SNARE complex, when VAMP-2 is introduced, it can easily displace the superfluous syntaxin molecule (Fasshauer et al., 1997). Additional candidates in the stabilization of the syntaxin 1a/SNAP-25 acceptor complex are the SM proteins, but this has yet to be fully substantiated (Toonen and Verhage, 2003).

N-terminal interaction between each neural SNARE proteins appears to facilitate SNARE complex assembly, which is important for vesicle priming and eventual membrane fusion (Borisovska et al., 2005; Pobbati et al., 2006). Unlike syntaxin 1a, the SNARE motifs found in monomeric SNAP-25 and VAMP-2 are largely unstructured, however, when combined, all four motifs will spontaneously interact and undergo tremendous conformational changes that lead to highly-organized structuring which provides the free energy required to drive membrane fusion (Hayashi et al., 1994; Fasshauer et al., 1997; Fasshauer et al., 2002). The energy released from this favorable interaction is derived from an increasing

numbers of hydrogen bonds and salt bridges that form during assembly, which locally reinforce the ionic and hydrophobic layers of the four-barreled helix, resulting in an extraordinarily stable core complex capable of resisting temperatures up to 80°C and concentrations of 8 M urea and 2% SDS.

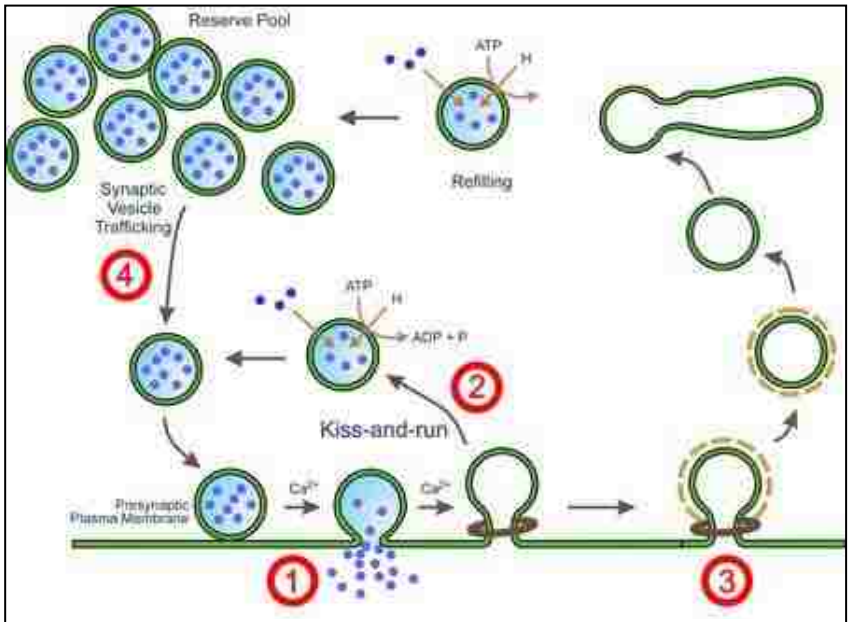
1.4 Membrane fusion

Neurotransmitter release requires targeting of synaptic vesicles to specialized fusion sites along the presynaptic membrane, defined as active zones (Landis et al., 1988; Hirokawa et al., 1989). Three vesicular pools exist with the synapse of a neuron: the largest and most distal, the reserve pool; an intermediately sized recycling pool comprised of vesicles that have been either recruited from the reserve pool or retrieved following fusion; and finally, a small pool of primed, or fusion-competent vesicles known as the readily releasable pool (RRP; reviewed in Turner et al., 1999). Vesicles within the reserve pool are tethered to the cytoskeletal infrastructure through an interaction with the protein synapsin. As calcium-triggered fusion occurs and depletes the RRP, vesicles can be recruited from the reserve pool via CAMKII phosphorylation of synapsin that results in the release of vesicles from the filamentous network (Fig. 1.3; Greengard et al., 1993). The vesicle will travel to the presynaptic membrane by first entering the recycling pool before finally being summoned to the active zone. Docking occurs via several protein-protein interactions that physically tether the vesicle to the plasma membrane; this step, however, does not provide the machinery

necessary for fusion. “Priming” is the process mediated by the SNARE complex assembly and the subsequent recruitment of proteins, such as the calcium sensor synaptotagmin I and voltage gated calcium channels (VGCCs), that create a fusion-competent vesicle (Littleton and Bellen, 1995; Seagar and Takahashi, 1998).

When a neuron is stimulated, an action potential will travel down the axon to the presynaptic terminal where membrane depolarization will result in the opening of VGCCs. This allows for the influx of calcium, raising local concentrations of this cation within microdomains proximal to the synaptic membrane. Because of their close interaction with VGCCs, calcium-triggered neuroexocytosis occurs quickly as conformational changes in both synaptotagamin and the SNARE complex drive membrane fusion. “Zippering” or

Figure 1.3 The Synaptic Vesicle Cycle



Schematic of vesicular fusion and cycling at the presynaptic terminal. **1**. Docking, priming, and calcium-triggered fusion of a synaptic vesicle. **2.** Vesicle entering rapid refill/reuse cycle known as “kiss-and-run.” **3.** Recapturing or recycling of synaptic membrane to create vesicles to be refilled. **4.** Synaptic vesicles are filled with neurotransmitter and stored in pools that can be readily recruited to the synaptic membrane. Reproduced from Greengard et al., 1993.

a progressive tightening of the SNARE complex is thought to occur from the N-terminal end of the four-barrelled helix to the C-terminal transmembrane domains of syntaxin 1a and VAMP-2, resulting in dramatic changes in free energy that drive membrane fusion (Hanson et al., 1997; Weber et al., 1998). Following fusion, SNAPs will bind NSF to the SNARE complex at which point an NSF-driven ATP-dependent reaction is carried out by NSF resulting in disassembly of the four-barreled helix (Sollner et al., 1993; Marz et al., 2003). Finally, through clathrin-dependent endocytosis, the vesicle will be reformed with its original protein content intact and enter into the recycling pool, where it may be refilled with neurotransmitter for a subsequent round of fusion, or eventually returned to the reserve pool.

1.5 Regulated expression of SNAP-25

Abundance of the t-SNARE SNAP-25 is regulated during brain growth and synaptogenesis at the level of expression and by the alternative splicing of a single gene (Bark et al., 1995). In the developing brain, two fundamental processes, axonal growth and synaptic plasticity, appear to coincide with changes in SNAP-25 expression. For example, SNAP-25 is detected in axonal growth cones of rat cortical neurons and inhibition of this protein with antisense oligonucleotides results in retardation of neurite elongation (Osen-Sand et al., 1993). In addition, dramatic increases in SNAP-25 expression levels accompany developmental stages marked by synaptogenesis and maturation of functional

synaptic connectivity (Catsicas et al., 1991). This expression is maintained in the mature mouse brain as SNAP-25 levels are highest in areas of increased synaptic plasticity (Oyler et al., 1989).

SNAP-25 is expressed as two different isoforms, SNAP-25a and 25b, which arise from developmentally-regulated alternative splicing between divergent, tandemly-arranged copies of a exon 5 (Bark, 1993). SNAP-25a mRNA is the major transcript expressed globally in the brain up until the third postnatal week at which point a significant change in the relative expression of the two isoforms occurs as SNAP-25b levels predominate and persist into maturity (Bark et al., 1995; Boschert et al., 1996; Jacobsson et al., 1999). However, adrenal chromaffin cells and other neuroendocrine cells do not undergo this dramatic developmental regulation and exclusively express SNAP-25a as the principle species into adulthood (Bark et al., 1995; Gonelle-Gispert et al., 1999; Grant et al., 1999). In addition, isoform expression also occurs differentially between anatomical regions of the adult mouse brain perhaps most striking seen in the reciprocal pattern of isoform expression characteristic of specific thalamic structures (Bark et al., 1995).

A short intron length between exons 5a and 5b results in a binary molecular switch wherein alternative splicing is obligatory and exclusionary, as inclusion of both exons into the mRNA would generate a codon frame shift and lead to a

truncated protein that is not likely to be functional. The alternatively spliced exons seemingly arose early in vertebrate evolution via duplication and are highly homologous, resulting in protein products that differ by only nine amino acid residues in mammals, including humans (Bark, 1993). These amino acid differences account for several non-conservative changes within the N-terminal helical SNARE domain as well as a rearrangement of the four centrally-located cysteine residues, that, when palmitoylated, allow for membrane association (Fasshauer et al., 1998).

Several reports have demonstrated distinct differences in function between 25a and 25b. In a transgenic mouse that maintains a higher SNAP-25a/b ratio throughout maturation, increased hippocampal short-term plasticity, as reflected by enhanced paired pulse facilitation, persists at levels similar to those found in juvenile brain (Bark et al., 2004). In addition, rescue of SNAP-25 deficient chromaffin cells with 25b instead of 25a, which is normally expressed in these cells, leads to an increased size of the primed, readily releasable pool of vesicles (Sorensen et al., 2003). When this experiment was repeated in *Snap25* null hippocampal neurons, 25b rescue once again resulted in a greater recruitment of primed vesicles for neuroexocytosis (Delgado-Martinez et al., 2007). Furthermore, the differential functional abilities of the two isoforms hinge on two important residues that reflect non-conservative, charge changes between SNAP-25a and SNAP-25b: H66Q (histidine to glutamine) and Q69K (glutamine

to lysine; Nagy et al., 2005). Structurally, when part of the four-barreled SNARE complex, these critical residues are arranged with their side chains facing externally, suggesting that differences in function are mediated by differential recruitment of accessory proteins, rather than an intrinsic effect on the SNARE complex itself.

Finding physiologically relevant interactions of SNAREs with candidate proteins is notoriously difficult due to the promiscuous binding of the alpha-helical motif. SNAP-25 is no exception and a multitude of binding partners have been reported but further mechanistic evidence remains absent. Results show that substantial changes in calcium dynamics occur relative to the expression levels of this SNARE, suggesting a valuable role for SNAP-25 in sculpting neurotransmission (Verderio et al., 2004). Functional relationships between this t-SNARE and proteins intimately involved in calcium-triggered vesicular fusion have been uncovered. For instance, SNAP-25, along with syntaxin 1a, interacts with (VGCCs), through what is termed a “synprint motif” indicating that these t-SNAREs are able to modulate calcium currents during membrane depolarization (Zhong et al., 1997; Catterall, 1999 for review). In addition, the discovery of interactions between SNAP-25 and synaptotagamin I, the major calcium sensor regulating neurotransmitter release, further supports the notion this t-SNARE represents a multifunctional protein involved in the control of secretion by multiple interactions (Schiavo et al., 1997; Zhong et al., 1997).

1.6 SNAP-25 expression and function in diverse neurotransmitter systems

The pivotal role of the neural t-SNARE SNAP-25 in evoked neurotransmitter release was originally identified through the use of Clostridial neurotoxins (CNT), a group of potent blockers of neurotransmission that consists of tetanus neurotoxin (TeNT) and seven botulinum neurotoxin serotypes (BoNT, A to G). Of the various CNTs, it is the BoNT/A and /E subtypes (and to a lesser extent, BoNT/C) that exclusively target SNAP-25, resulting in differential site-specific cleavage of 9 and 26 residues, respectively, from the C-terminus of this SNARE, which abolishes the activity-dependent release of a wide variety of transmitters, including acetylcholine, glutamate, catecholamines, and various peptides, such as insulin and growth hormone (Graham et al., 2002; for review, see Montecucco et al., 2005).

Explanation of *Snap25* null mutant mice support the findings reported from the earlier studies using neurotoxin blockades. Specifically, neurons and neuroendocrine cells devoid of SNAP-25 lack evoked neurotransmission in catecholaminergic, glutamatergic, and cholinergic systems (Washbourne et al., 2002; Sorensen et al., 2003). As with BoNT intoxication, specific loss of action potential (AP)-dependent transmission occurs in *Snap25* null mice, however mutant neurons and chromaffin cells retain stimulus-independent transmitter release. Taken together, results from SNAP-25 knockout and BoNT treated cells indicate that SNAP-25 is part of a SNARE complex that is fundamental for

the evoked release of many different neurotransmitters; however, AP-independent vesicular fusion is carried out by an alternative SNAP-25-independent SNARE complex.

It is interesting, however, that while spontaneous vesicular fusion persists in the absence of SNAP-25, the frequency and amplitude of these events are altered differently between neurons of various neurotransmitter phenotypes. For SNAP-25 knockout cholinergic terminals at the neuromuscular junction, the frequency and amplitude of TTX-resistant miniature release events ("minis") are increased, unlike glutamatergic neurons that display a decrease solely in frequency (Washbourne et al., 2002). Additionally, when SNAP-25 is cleaved with botulinum neurotoxin in glutamatergic neurons, similar decreases in miniature excitatory postsynaptic current (mEPSC) frequency are observed in glutamatergic neurons (Capogna et al., 1997). Therefore, while SNAP-25 is not required for constitutive neurotransmitter release, it may play a modulatory role in AP-independent vesicular fusion. Furthermore, due to differential effects of SNAP-25 ablation in various neurotransmitter systems, this t-SNARE and its isoforms may provide a flexible scaffold on which to build discrete fusiogenic machinery tailored to suit the neurotransmitter phenotype of the cell.

1.7 The role of SNAP-25 in inhibitory neurotransmission

As outlined in the previous section, the requirement for SNAP-25-containing SNARE complexes for evoked neuroexocytosis has been demonstrated in several cell types and excitatory neurotransmitter systems. Therefore, the hypothesis proposed for this dissertation project is that SNAP-25 is part of a universal neural fusiogenic core complex required for AP-dependent neuroexocytosis regardless of neurotransmitter phenotype. Thus, it was expected that expression of this t-SNARE should extend to GABAergic neurons and mediate calcium-triggered inhibitory transmission. However, the few early reports published regarding the involvement of SNAP-25 in evoked GABAergic transmission were conflicting and this topic remained debatable. Therefore, in order to fully convey the rationale behind the experiments conducted for this project, which will be described in the following chapters of this dissertation, it is necessary to provide the historical context from which this hypothesis arose.

At the time our investigations began, most of the evidence supporting a fundamental role for SNAP-25 in regulated neuroexocytosis had been acquired primarily on studies that focused on either excitatory neurotransmitters or neuropeptides. Evidence extending the existence of a SNAP-25-dependent mechanism for evoked neurotransmission in inhibitory neurons was limited to two studies. Using BoNT/A and BoNT/E neurotoxin blockades, one group had shown that the evoked release of glycine, a major inhibitory neurotransmitter in the

brainstem and spinal cord, was abolished in cultured neurons (Keller et al., 2004). However, correlative evidence for SNAP-25 involvement in GABAergic transmission was restricted to a single study showing that, following BoNT/E treatment, isolated rat synaptosomes failed to release GABA during potassium-induced depolarization (Ashton and Dolly, 2000). While this study provided good biochemical evidence for the mechanistic requirement for SNAP-25 in evoked GABA release, the assay used purified rat synaptosomes and did not address GABA release under more physiological conditions from intact neurons connected within a functional circuit.

In contrast, a surprising report was published that challenged the universal neural SNARE model by proposing that SNAP-25 was not expressed in GABAergic neurons, and therefore, inhibitory neurotransmission occurred via an alternative neural SNARE complex (Verderio et al., 2004). Using dual stain immunohistochemistry for both SNAP-25 and a GABAergic marker, Verderio and colleaques reported that SNAP-25 immunoreactivity was undetectable in GABAergic neurons surveyed in either hippocampal cultures or adult tissue sections. In an accompanying vesicular recycling assay, this study showed that stimulus-dependent exo/endocytosis in GABAergic synapses was not blocked during BoNT/A treatment. However, the same group published a subsequent and somewhat conflicting report that showed SNAP-25 immunoreactivity in cultured GABAergic neurons; albeit with continuous reductions in signal intensity

over the course of 10 days of growth coincident with increased insensitivity of these synapses to BoNT/A intoxication (Frassoni et al., 2005). The conclusion reached by Frassoni and colleagues was that SNAP-25 is only transiently expressed and utilized by immature GABAergic neurons and that another SNARE, perhaps SNAP-23, was responsible for activity-dependent neurotransmitter release at inhibitory terminals.

Historically, BoNT blockade assays are notorious for providing variable results as neurotoxin activity can be greatly altered as a result of factors such as the calcium concentration present in experimental buffers (Capogna et al., 1997; Huang et al., 2001). Therefore, exploiting a mouse model with a targeted genetic deletion provides a reasonable alternative for determining SNAP-25's role in GABAergic transmission. In collaboration with our lab, Manuel Mameli and Fernando Valenzuela conducted initial electrophysiological studies in neurons of fetal *Snap25* null mice. Using patch-clamp recordings, they demonstrated that ablation of SNAP-25 eliminated evoked GABAA receptor-mediated postsynaptic responses while sparing the spontaneous AP-independent events, supporting the requirement of SNAP-25 in the Ca^{2+}-triggered synaptic transmission of early developing GABAergic neurons (data included in Chapter 2 and published as Tafoya et al., 2006).

1.8 Does the exocytotic machinery in GABAergic neurons have unique properties?

GABAergic neurons possess an electrophysiological profile that displays exclusive activity patterns suited to operate with high-speed, reliability, and precision. This includes, for example, the use of both electrical coupling and chemical neurotransmission, fast spike properties with low spike latencies, a high calcium influx upon depolarization, and a slightly depolarized resting membrane potential for fast activation upon excitation compared to other neurons {reviewed by Jonas 2004, Verderio et al., 2004}. A distinctive signature of neurotransmission, therefore, could be the result of customization of the fusogenic machinery at the presynaptic terminal to maximize attributes such as calcium sensitivity. This could be achieved through the differential expression by GABAergic neurons of presynaptic proteins that participate in vesicular fusion.

Recent evidence supports these possibilities for differences in the GABAergic system since the presynaptic proteins Munc13-1, RIM1α (Rab3-interacting molecule 1α), and synapsin exert different modulatory affects on glutamate and GABA neurotransmission (Augustin et al., 1999; Schoch et al., 2002; Gitler et al., 2004), indicating that a specific constellation of protein effectors might be used during GABA release. It stands to reason that customization could result from the differential expression of core complex components, which would create

unique scaffolds to recruit defined groups of accessory proteins to the neuroexocytotic machinery.

The expression of SNAP-25 as two functionally distinct isoforms suggests this t-SNARE as a candidate for involvement in such a mechanism (Sorensen et al., 2003; Bark et al., 2004; Delgado-Martinez et al., 2007). In addition to neuroendocrine cells, neurons within some distinct neuroanatomical regions of the adult brain maintain exclusive expression of 25a isoform, suggesting that the ratio of SNAP-25 isoform expression might contribute to the diversity of transmission in mature, established synapses within neural networks (Bark et al., 1995; Boschert et al., 1996; Jacobsson et al., 1996; Gonelle-Gispert et al., 1999; Grant et al., 1999; Jacobsson et al., 1999a; Jacobsson et al., 1999b). The goals of my project include, amongst others, the investigation into this possibility and are outlined below.

1.9 Goals of this investigation:

Due to the conflicting reports discussed in the previous section and based on findings in several other neurotransmitter systems, consistent with a role for SNAP-25 in a universal neuroexocytotic mechanism, I propose the following hypothesis: GABAergic neurons require SNAP-25 dependent facilitation of calcium-triggered neurotransmission and maintain SNAP-25a expression into

adulthood. In order to investigate this issue, I will examine the expression, function, and possible developmental regulation of SNAP-25 in GABAergic neurons as outlined in the following specific aims:

Specific Aim 1: To determine the expression and cellular localization of SNAP-25 in fetal and adult GABAergic neurons.

Because reports from Verderio (2004) and Frassoni (2005) suggesting that GABAergic neurons are devoid of SNAP-25, this study will first determine the expression and sorting of this t-SNARE to GABAergic terminals. Establishing the expression and proper cellular localization of SNAP-25 to the presynaptic terminal is crucial, as this is the site of action for SNARE complexes during neurotransmitter release. Chapter 2 will show the results of immunostaining and fluorescent in situ hybridization (FISH) experiments that demonstrate persistent SNAP-25 expression and appropriate presynaptic localization in both fetal and adult GABAergic cells that is comparable to glutamatergic neurons.

To briefly outline the experiments addressing this aim, I first performed a dual stain immunohistochemistry experiment on DIV 9-21 cultured neurons using antibodies specific to SNAP-25 and either the vesicular transport protein for GABA (VGAT) or glutamate (VGLUT1). In order to distinguish and resolve colocalization of punctate immunostaining to the same terminal, I used

dissociated hippocampal cultures, which develop extensive processes and fine synaptic networks. A subsequent immunostaining experiment was then conducted on adult coronal brain sections using the same antibody combinations. Finally, I performed FISH analysis on adult brains sections using probes to detect the GABAergic marker GAD65/67 and SNAP-25 mRNA. I surveyed four different anatomical regions and provide evidence for mRNA expression of SNAP-25 in all GABAergic neurons within the brain.

Specific Aim 2: To determine whether stimulus-evoked, but not action potential-independent, vesicular recycling is abolished in both glutamatergic and GABAergic synapses of *Snap25*$^{-/-}$ neurons.

Mameli and Valenzuela provided electrophysiological data showing a lack of evoked GABAergic transmission and decreased AP-independent spontaneous activity in fetal SNAP-25 deficient brains (Tafoya et al., 2006). These recordings of postsynaptic responses provide only indirect information about presynaptic mechanisms. These postsynaptic responses could be altered through compensatory mechanisms as the brain develops in the absence of evoked synaptic activity. Thus, in crude synaptosomal fractions prepared from E17.5 *Snap25*$^{-/-}$ mutant and control forebrain, we used Western blotting to confirm that alterations in GABAergic transmission were due to the absence of a SNAP-25-dependent release mechanism rather than a reduction in synaptic vesicle number/neurotransmitter content (presented in Chapter 2; Tafoya et al., 2006).

Because SNAP-25 deletion is lethal perinatally, any brain slice recordings are limited to mutant neurons at the E17.5 fetal stage. Conseqently, SNAP-25 function cannot be assessed in the adult brain; however, culturing *Snap25*$^{-/-}$ neurons allows for extended development and maturation of synapses. Furthermore, to determine the functional role of SNAP-25 in GABAergic vesicular recycling, we focused on SNAP-25 knockout neuronal cultures grown for 10-12 days in vitro (DIV), performing an FM 1-43FX styryl dye uptake assay at immunolabeled GABAergic and glutamatergic presynaptic terminals in order to directly measure AP-dependent and –independent vesicular recycling. Results showed that stimulus evoked, but not AP-independent, coupled exo/endocytosis was equally blocked in both VGAT- and VGLUT1-immunopositive synapses and are described fully in Chapter 2 (Tafoya et al., 2006) and Chapter 3 (Tafoya et al., 2008), respectively.

Specific Aim 3: To determine the expression pattern of SNAP-25 isoforms in GABAergic neurons.

In order to complete an extensive evaluation of SNAP-25 in GABAergic cells, developmental and neuroanatomical regulation of isoform expression in the major inhibitory neurons of the juvenile and adult brain should be determined. If present, differences between GABAergic and glutamatergic neurons in the relative levels of SNAP-25a and 25b could provide novel insight into the molecular machinery tailored for the unique properties of inhibitory and excitatory

neurotransmission. Therefore, the final set of experiments completed explored whether differential expression of the functionally distinct isoforms of SNAP-25 occurs between several different populations of GABAergic and glutamatergic neurons.

In experiments using GAD67-eGFP(ΔNeo) mice (Tamamaki et al., 2003), which express green fluorescent protein in virtually all GABAergic populations, I isolated pure populations of mature GFP-positive neurons through single cell microdissections of four different anatomical regions via laser capture microscopy (LCM). In addition, I purified GFP-positive neurons from juvenile and adult mice using fluorescence-activated cell sorting (FACS). In both experiments, as glutamatergic controls I also harvested GFP-negative, largely excitatory, neuronal populations. cDNA was synthesized from total RNA extracts of each sample and the level of isoform expression was determined using quantitative RT-PCR (qRT-PCR) with β–actin (housekeeping gene used for normalization) and specific primer sets to distinguish SNAP-25a and 25b transcripts. Detailed findings from these investigations can be found in Chapter 3 (Tafoya et al., 2008).

2. Expression and Function of SNAP-25 as a Universal SNARE Component in GABAergic Neurons

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2.1 Abstract

Intracellular vesicular trafficking and membrane fusion are important processes for nervous system development and for the function of neural circuits. SNAP-25 is a component of neural SNARE core complexes that mediates the exocytotic release of neurotransmitters at chemical synapses. Previous results from mouse mutant models and pharmacological/neurotoxin blockades have demonstrated a critical role for SNAP-25-containing SNARE complexes in action potential (AP)-dependent release at cholinergic and glutamatergic synapses and for calcium-triggered catecholamine release from chromaffin cells. To examine whether SNAP-25 participates in the evoked release of other neurotransmitters, we investigated the expression and function of SNAP-25 in GABAergic terminals. Patch-clamp recordings in fetal *Snap25* null mutant cortex demonstrated that ablation of SNAP-25 eliminated evoked GABAA receptor-mediated postsynaptic responses while leaving a low level of spontaneous AP-independent events intact, supporting SNAP-25's involvement in the regulated synaptic transmission of early developing GABAergic neurons. In hippocampal cell cultures of wild type mice, punctate staining of SNAP-25 colocalized with both GABAergic and glutamatergic synaptic markers, whereas stimulus-evoked vesicular recycling was abolished at terminals of both transmitter phenotypes in *Snap25*$^{-/-}$ neurons. Moreover, immunohistochemistry and fluorescent in situ hybridization revealed co-expression of SNAP-25, GABA vesicular transporter (VGAT), and GAD65/67 in interneurons within several regions of the adult brain. Our results thus provide evidence that SNAP-25 is critical for evoked GABA release during development

and is expressed in the presynaptic terminals of mature GABAergic neurons, consistent with its function as a component of a fundamental core SNARE complex required for stimulus-driven neurotransmission.

2.2 Introduction

SNARE protein complexes are essential in promoting vesicular fusion for neurotransmission (Sudhof, 2004). While the precise mechanisms of SNARE function remain unidentified, it is recognized that this family of proteins (Fasshauer et al., 1998; Bock et al., 2001) interact through amphipathic SNARE domains to create a four-helix bundle that is capable of bridging apposing membranes prior to fusion (Sutton et al., 1998). Different SNARE complexes appear to be involved in virtually all elements of vesicular trafficking, including intracellular transport, constitutive and stimulus-driven delivery of plasma membrane proteins, as well as regulated neuroexocytosis (see, Jahn et al., 2003; Hong, 2005). In neurons, SNARE complex composition may help distinguish process outgrowth during development from neurotransmitter release (Bark and Wilson, 1994; Martinez-Arca et al., 2001; Washbourne et al., 2002). Besides acting directly in membrane fusion, SNARE complexes may also provide a scaffold to recruit accessory proteins that contribute distinct physiological properties required for various fusion events (Melia et al., 2002; Nagy et al., 2005)

The predominant neural SNARE complex is composed of plasma membrane-associated SNAP-25 and syntaxin 1a, and vesicular membrane-associated synaptobrevin (Sollner et al., 1993). The involvement of these SNARE proteins in neuroexocytosis has been addressed using the proteolytic tetanus and botulinum neurotoxins (BoNT) that block neurotransmission (for review, see Montecucco et

al., 2005). In particular, BoNT/A and /E target SNAP-25, disrupting activity-dependent release of various transmitters and peptides (Sadoul et al., 1995; Graham et al., 2002). Recently we have provided genetic evidence that SNAP-25 is critical for evoked glutamatergic and cholinergic transmission in central neurons and at neuromuscular junctions (Washbourne et al., 2002), and for fast calcium-triggered catecholamine release from adrenal chromaffin cells (Sorensen et al., 2003). Nevertheless, *Snap25* null mutants retained stimulus-independent transmitter secretion (Washbourne et al., 2002; Sorensen et al., 2003), suggesting that alternative SNARE constituents could promote spontaneous vesicle fusion and transmitter release. However, it has yet to be established what importance SNAP-25-independent transmission might have in neural communication, for example during brain development (Molnar et al., 2002).

Recent evidence suggests that the presynaptic proteins Munc13-1, RIM1α, and synapsin exert different modulatory affects on glutamate and GABA neurotransmission (Augustin et al., 1999; Schoch et al., 2002; Gitler et al., 2004), and that these accessory proteins may contribute to shaping physiological parameters that govern synaptic activity. Although SNAP-25 appears to be required for glycine release from spinal interneurons (Keller et al., 2004), it has been suggested that SNAP-25 is not expressed by central GABAergic neurons and that inhibitory transmission may occur without this neural SNARE protein (Verderio et al., 2004; Frassoni et al., 2005). Given the implication that calcium-

triggered inhibitory and excitatory neurotransmission could be distinguished presynaptically by interchanging core components of the SNARE complex, we sought to examine whether SNAP-25 is required for GABA release in *Snap25* null mutant fetal brain and whether it is expressed in developing and adult GABAergic neurons. Our findings indicate that SNAP-25 is critical for GABAergic transmission and support its role as a primary constituent of SNARE complexes required for stimulus-evoked neuroexocytosis.

2.3 Materials and Methods

Electrophysiology

Snap25 null mutant mice were collected through a timed pregnancy protocol from heterozygous matings wherein pregnant animals (plug date = day 0) were sacrificed by rapid cervical dislocation and decapitation. Fetuses were removed sequentially from the uterus, and tested for movement by a brief pinch to the hind limb. As *Snap25* null homozygote mutant animals lack evoked neuromuscular transmission (Washbourne et al., 2002), fetuses were initially categorized based on the presence of movement, with subsequent PCR genotyping to confirm knockout animals, and to distinguish between heterozygote and homozygote wild type mice that served as control littermates. Pups were quickly decapitated and their brains were removed and placed in ice cold PBS. All procedures were performed in accordance with guidelines of the University of New Mexico Health Sciences Center Laboratory Animal Care and Use committee, and the National Institutes of Health.

Coronal brain slices (350 to 400 µm) were prepared from wild type and mutant mice at embryonic day 17.5 as previously described (Carta et al., 2004), except that ketamine was not used. After a recovery period of ≥80 min, slices were transferred to a chamber perfused at rate of 2 ml/min with artificial cerebrospinal fluid (ACSF) equilibrated with 95% O2 / 5% CO2 and containing in (mM): 126 NaCl, 3 KCl, 1.25 NaH2PO4, 1 MgSO4, 26 NaHCO3, 2 CaCl2 and 10 glucose.

When indicated, 20 µM bicuculline methiodide was added to the ACSF to block GABAA receptors. Whole-cell patch-clamp electrophysiological recordings from visualized cortical neurons were performed under infrared-differential interference contrast microscopy at 32˚C, with an Axopatch 200B amplifier (Axon Laboratories, Union City, CA). Patch electrodes had a resistance of 3-5 MΩ. For voltage-clamp experiments, patch pipettes were filled with an internal solution containing in mM: 140 CsCl, 2 MgCl2, 1 CaCl2, 2 Na2-ATP, 10 EGTA, and 10 HEPES at pH 7.3. Current-clamp experiments (Iholding = 0) were conducted with patch pipettes filled with an internal solution containing in mM: 135 K-gluconate, 10 MgCl2, 0.1 CaCl2, 2 Na2-ATP, 1 EGTA, and 10 HEPES at pH 7.3 Access resistance was between 10-30 MΩ; if access resistance changed >20%, the recording was discarded. Spontaneous postsynaptic currents (sPSC) were recorded at a holding potential of -65 mV in the presence of 10 µM NBQX (Axxorra, San Diego, CA), and 100 µM D, L-APV. Miniature PSCs (mPSCs) were recorded in presence of 500 nM tetrodotoxin (Calbiochem, San Diego, CA), 10 µM NBQX, and 100 µM D, L-APV. Evoked PSCs (ePSCs) were recorded in presence of NBQX and D, L-APV with 4 mM QX-314 in the internal pipette solution and were elicited with a concentric bipolar stimulating electrode placed 100-200 µm from the patched cell. Data were acquired and analyzed with pClamp-9 (Axon Laboratories). Minis Analysis program (Synaptosoft, Decatur, GA) was used to analyze mPSCs. Statistical analyses of pooled data were performed by unpaired Student's t-test (Prism 4; Graphpad Software, San Diego, CA). All values were expressed as mean ± SEM.

Protein analysis

For protein analysis, E17.5 mouse brains were removed and immediately immersed in ice-cold PBS. Hippocampi and cortices were then dissected over ice, homogenized in 0.32 M sucrose, 20 mM Tris pH 7.4, 1 mM DTT, 1% Nonidet P-40 and protease inhibitors (Complete Minitablet, Boehringer-Mannheim, Indianapolis, Indiana), and processed through differential centrifugation to obtain LP2 protein fractions as described previously (Huttner et al., 1983). Protein concentrations of the protein samples were determined with Micro BCA assay kit (Pierce, Rockford, Illinois) and equal amounts of protein were loaded on 12% SDS-PAGE polyacrylamide gels with subsequent transfer to PVDF membrane (BioRad, Hercules, CA). Blots were then probed with the antibodies to the following proteins: synaptophysin (1:1500, Invitrogen Corporation, Carlsbad, CA), VGLUT1 (1:500, Synaptic Systems, Göttingen, Germany), and VGAT (1:200, Synaptic Systems). Primary antibodies were detected with species-appropriate HRP-conjugated secondary IgGs followed by chemiluminescence assay (ECL-Plus, Amersham Pharmacia, Piscataway, New Jersey) and quantitated on a STORM PhosphoImager system (Molecular Dynamics, Sunnyvale, California). One-way ANOVA analysis on the data was carried out with Bonferroni comparisons post hoc test (Prism 4). All values expressed as mean ± SEM.

FM 1-43FX vesicular recycling assay

Hippocampal neurons were isolated from individual E17.5 fetal mice, genotyped as detailed above, and grown as dispersed mixed cell cultures as described previously (Washbourne et al., 2002). Briefly, hippocampi were microdissected in ice cold PBS containing 27 mM glucose, 17.5 mM sucrose, and 15 mM HEPES with a final pH of 7.4. After incubation with 0.05% trypsin-EDTA (Invitrogen) for 10 minutes at 37°C the hippocampi were then transferred to Neurobasal A media (Invitrogen) supplemented with 10% FBS, 25 μM glutamate, 0.5 mM L-glutamine, and 1% penicillin/streptomycin and gently triturated with flame polished Pasteur pipettes. The dispersed cells were plated on poly-L-lysine/collagen coated 12 mm coverslips (four coverslips per animal, ~50,000 cells/coverslip). For the first 24 hours, cultures were grown in the supplemented media described above. The media was then replaced with Neurobasal A media containing B27 supplement (Invitrogen) instead of FBS, and after 5 days in vitro (DIV 5), glutamate was removed entirely from the culture media. Every third day beyond this point, 1/3 of the media was removed and replaced with Neurobasal A media containing B27 supplement without glutamate.

To assay FM 1-43FX dye uptake, coverslips of DIV 12 neurons were briefly washed in PBS (37°C) and then incubated for 15 minutes in modified Tyrode's solution (150 mM NaCl, 4 mM KCl, 2 mM MgCl, 10 mM glucose, 10 mM HEPES, and 2 mM CaCl2, pH 7.4). During this incubation and throughout the duration of

the experiment, all buffers were used at 37°C and contained 10 µM CNQX, 25 µM APV, 20 µM bicuculline, and 0.6 µM TTX to prevent spontaneous action potential-dependent neuronal activity. Synaptic vesicles were loaded in the presence of 15 µM FM1-43FX dye (Molecular Probes, Eugene, OR) after evoking exocytosis with either high K^+ (64 mM NaCl, 90 mM KCl, 2 mM MgCl, 10 mM glucose, 10 mM HEPES, and 2 mM CaCl2, pH 7.4) for 90 seconds or hypertonic sucrose (500 mOsm added to modified Tyrode's solution) for 30 seconds (Sara et al., 2002). Cells were then immediately washed in modified Tyrode's solution for 15 minutes and either fixed with 4% paraformaldehyde/4% sucrose or destained in high K^+ solution for 2 minutes in the absence of dye before washing and fixation.

Fixed cultures were immunostained with either VGAT or VGLUT1 monoclonal antibodies (1:1000, Synaptic Systems) followed by Alexa 647 conjugated anti-mouse secondary antibody (Molecular Probes) and then visualized with a Zeiss LSM 510 META/Axiovert 100M laser confocal microscope using a 63x oil DIC objective (n.a. = 1.4). META photodetectors were configured to recognize fluorescent emissions within the spectral range of 556-716 nm, and the peak emissions of FM 1-43FX (598 nm) and Alexa 647 (663 nm) were captured at 585-609 nm and 652-673 nm, respectively. FM 1-43FX fluorescence intensity that colocalized within the immunoreactive punctate staining of either VGAT or VGLUT1 was measured by using the mean ROI intensity function of the LSM 510

bundled software. A region of interest (ROI) corresponds to a single point of five pixels that overlays the majority of area within each puncta. Areas of low neurite density were chosen to reduce the chance of incidental colocalization of the punctate vesicular transporter stain with non-synaptic FM1-43FX membrane staining distributed along neurite fibers. Ten puncta present on no less than three neurites were measured per field, averaged, and used as a single value for each animal (n= 5 animals per genotype and experimental condition). After subtraction of background fluorescence, data was analyzed using one-way ANOVA with Bonferroni post hoc comparisons (Prism 4). All values expressed as mean ± SEM. Although brightness and contrast was adjusted for clarity in images used in figures (Adobe Photoshop 7.0 software; Adobe Systems Inc., San Jose, CA), all quantitative analysis was done on raw data before any manipulation of the image.

Fluorescent Immunocytochemistry

After 9-21 days in culture, E17.5 hippocampal neurons were washed in PBS at 37°C followed by a 5 minute fixation in 4% paraformaldehyde (PFA)/Sucrose (37^{o}C) in 0.1 M phosphate buffer (PB) at 37^{o}C. A second round of washing in warm PBS was followed by a five minute incubation of the cover slips at room temperature in a quenching solution (0.38% glycine, 0.27% NH4Cl). Finally, the cover slips were stored at 4^{o}C in PBS containing 0.02% sodium azide.

For tissue collection, adult mice (> PN 50) were anesthetized with pentobarbital (50 µg/gm) and perfused transcardially with 30 ml of 0.1 M phosphate buffer (PB) followed by 30 ml of 4% PFA in 0.1 M PB. Brains were dissected and then post-fixed in 4% PFA/0.1 M PB overnight at 4°C, followed by immersion in 30% sucrose for 48 prior to cryosectioning. Tissue was then frozen in 30% sucrose/0.1 M PB solution before sectioning. Thirty micron coronal sections were cut using a sliding microtome.

Prior to applying antibodies, both cultured cells and brain sections were permeabilized and non-specific antibody interactions were blocked by incubating in 1% donkey serum, 1% tween-20, in Tris-buffered saline (TBS). Primary antibodies were used at the following dilutions: GAD 65/67 rabbit polyclonal antibody (1:200, a generous gift from Michele Solimena, Dresden University of Technology, Dresden, Germany), and both mouse monoclonal and rabbit polyclonal antibodies to VGLUT1 and VGAT (1:1000; C1 317D5 and C1 117G4, respectively, Synaptic Systems). For the experiments described, we used the SNAP-25 monoclonal antibody SMI 81 (1:1000; Sternberger Monoclonals, Lutherville, MD). The specificity of this antibody was demonstrated by its robust reactivity to a single 25 kDa protein band in wild type brain, but also through the absence of detectible immunoreactivity either by western blot or immunofluorescence in SNAP-25 knockout mutant neurons (Fig. 2.1). These criteria, in our hands, were not satisfactorily fulfilled by all SNAP-25 antibodies

(data not shown). Coverslips and sections of fixed culture and tissue samples were then incubated with Cy™3-conjugated affinipure donkey anti-rabbit IgG (H+L) (1:1000, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and Alexa Fluor® 488 donkey anti-mouse IgG antibodies (1:200, Molecular Probes) as secondary antibodies for species-appropriate detection of primary antibodies. Finally, To-Pro®-3 iodide (Molecular Probes) was used for nuclear counterstaining.

Images of the immunofluorescent staining were obtained using a BioRad 2100 Radiance confocal microscope using a 63X oil immersion DIC objective (n.a.=1.4) where x, y pixel corresponds to 0.2 µm per pixel with a resolution of 1024 X 1024. Under these conditions, our z axis, or optical slice, was 0.8 µm. Before viewing double-stained material, we calibrated the appropriate laser intensity, amplifier gain, and signal offset settings for each marker with single-stained specimens in order to prevent saturation of their respective signals. Our confocal settings were optimized to acquire the linear range of fluorescence signal in our desired regions of interest, which in some cases may have resulted in slight pixel saturation in neighboring regions containing higher levels of synaptic density. In addition, our data was quantitated and collected prior to any manipulation of brightness or contrast that would compromise the integrity of pixel intensity. Punctate colocalization of separate color channels was then isolated and quantitated using comparable threshold level adjustments

(Metamorph 6.1 software, Universal Imaging Corporation, Downingtown, PA). In short, this analysis did not count individual puncta, but rather, compared the total pixel number of each stain and calculated the percentage or degree of their overlap. Due to differing sizes of punctate fluorescence between stains, not all pixels overlapped, resulting in percentages below 100%. It is important to note the percentages, therefore, do not reflect the percentage of synapses that are double positive, but corresponds to the total pixel overlap across each field. Each colocalization value was determined using the average of 3 fields per animal (n = 3 animals). Statistical analysis of colocalization was performed using one-way ANOVA with Bonferroni post hoc comparisons using Prism 4 software. All values were expressed as mean ± SEM.

Fluorescence In Situ hybridization

Adult mice (PN>50) were euthanized with isoflurane and decapitated. Brains were quickly removed and quick-frozen in a beaker of isopentane equilibrated in a dry ice/ethanol slurry and stored at −70°C until further processing. Coronal brain sections (20 µm) were prepared using a cryostat and arranged on slides (Superfrost Plus, VWR) that were air dried and stored frozen at −20°C until use.

Double-labeled fluorescence in situ hybridization (FISH) for SNAP-25 and GAD65/67 mRNA was performed as described in detail previously (Guzowski et

al., 1999; Vazdarjanova et al., 2002; Vazdarjanova and Guzowski, 2004). Briefly, digoxigenin-labeled SNAP-25 and fluorescein-labeled GAD65/67 antisense riboprobes were generated from cDNA plasmids using a commercial transcription kit (MaxiScript; Ambion, Austin, Texas) and premixed RNA labeling nucleotide mixes containing either digoxigenin or fluorescein-labeled UTP (Roche Molecular Biochemicals). Hybridization with the tissue was carried out overnight at 56°C. Then, the digoxigenin-labeled SNAP-25 riboprobe was detected with anti-digoxigenin–HRP conjugate (Roche Applied Science, Indianapolis, IN), amplified with a TSA-Biotin kit (PerkinElmer Life Sciences, Emeryville, CA) and detected with streptavidin-Cyanine-3 (Jackson Labs)). Subsequently, the slides were then treated with 2% H2O2 to quench any residual HRP activity, and the fluorescein-labeled probe GAD65/67 was detected with an anti-fluorescein-HRP conjugate (Roche Applied Science) and a TSA-FITC substrate kit (FITC Direct FISH; PerkinElmer Life Sciences). Nuclei were counterstained with DAPI (Molecular Probes, Eugene, OR).

Images were acquired with a Nikon TE2000U epifluorescence microscope with a 20X dry objective (NA = 0.75) and captured using a CoolSNAP-Hq CCD Camera (Roper Scientific). Images of DAPI (cell nuclei), CY3 (SNAP-25), and FITC (GAD65/67) were acquired and color-combined using Metamorph software (Universal Imaging). The images were analyzed by counting the total number of GAD65/67-positive neurons and then determining the percentage of those that

were double positive for SNAP-25 fluorescence using Image J software (made available as freeware through the National Institutes of Health). Statistical analysis was carried out through one-way ANOVA with Bonferroni comparison using Prism 4 software. All values were expressed as mean % ± SEM per region.

2.4 Results

SNAP-25 is crucial for GABAergic transmission in fetal neurons.

To assess whether SNAP-25 plays a role in GABAergic neurotransmission in fetal brain slices, we conducted whole-cell patch-clamp recording experiments in cortical slices from *Snap25* null mutants (Washbourne et al., 2002) and control E17.5 fetal mice. This homozygous null mouse mutant does not possess a functional *Snap25* gene and allows us to investigate the specific role that SNAP-25 may play in neurotransmission. While mice homozygous for this mutation develop normally during gestation, these mutants die upon birth at least in part due to a lack of cholinergic transmission to the diaphragm, resulting in respiratory failure.

Cortical pyramidal neurons were visualized with infrared microscopy, then randomly patched and recorded in the presence of the NMDA and AMPA receptor antagonists (100 μM D, L-APV and 10 μM NBQX, respectively) to isolate GABAergic currents. In these whole-cell patch-clamp experiments, the observed evoked postsynaptic currents (ePSCs) were obtained at a holding potential of -65 mV evoked by stimulation of 100-150 pA for 100 μs with a bipolar electrode positioned near the target cell. As previously, recordings from wild type and heterozygote *Snap25* null mutant littermates were indistinguishable and thus used as controls for all electrophysiological experiments (Washbourne et al., 2002; Sorensen et al., 2003). Illustrated in Figure 2.2A, ePSCs were repeatedly

and consistently triggered in control (n=9) neurons after stimulation, whereas mutant (n=8) cells showed an absence of evoked transmission. Treatment with the selective $GABA_A$ receptor antagonist bicuculline (20 μM; panel A, dotted trace) abolished the ePSCs in control slices, confirming that these events are mediated by $GABA_A$ receptors.

We next examined spontaneous GABAergic transmission by recording transient inward currents at a holding potential of -65 mV both with and without the administration of tetrodotoxin (TTX). As shown in Figure 2.2B-C, spontaneous postsynaptic currents (sPSCs) observed in SNAP-25 deficient slices were significantly reduced in both frequency and amplitude when compared to controls. The frequency was reduced 26-fold from 1.36±0.46 Hz for control neurons to 0.05± 0.03 Hz for mutant neurons. The amplitude of sPSCs was reduced 6-fold from 71.0±12.0 pA for control neurons to 11.8± 2.96 pA for mutant neurons. Recordings were then made after application of TTX (0.5 μM) to block action potential propagation and isolate miniature postsynaptic currents (mPSCs). As depicted in Figure 2.2D-E, *Snap25*$^{-/-}$ mutant neurons still exhibited detectable levels of TTX-resistant GABAergic mPSCs. Similar to recordings of sPSCs, there was a significant reduction in the frequency and amplitude of mPSCs (0.06±0.03 Hz, 11.2±1.59 pA) from mutant neurons compared to those recorded from control neurons (0.73±0.13, 57.7±10.8 pA). Interestingly, TTX treatment did not significantly reduce the amplitude or frequency of either sPSC

or mPSC events in *Snap25* null mutants, whereas neurons from control slices showed a reduction in both amplitude (18.7%) and frequency (46%) of spontaneous activity when action potential propagation is inhibited. This is consistent with the complete loss of AP-dependent, evoked GABAergic transmission in the absence of SNAP-25, as reported for glutamatergic and cholinergic synapses (Washbourne et al., 2002). Treatment with bicuculline blocked both sPSC and mPSC activity in control and mutant slices, demonstrating that GABAA receptor activation mediated these spontaneous events.

The reduced level of GABAergic transmission in SNAP-25 deficient neurons may have been due to decreased postsynaptic GABA responsiveness, possibly by the down-regulation of $GABA_A$ receptors. To examine $GABA_A$ receptor function in *Snap25*$^{-/-}$ neurons, we recorded inward currents at a holding potential of -65 mV evoked by exogenous application of GABA (50 µM) in the presence of TTX (0.5 mM). *Snap25* null mutant slices displayed a robust response that exceeded that of the control by over 8-fold (-10.96±1.43 pA/pF and -1.30±0.45 pA/pF, respectively, Fig. 2.2F), consistent with a potential up-regulation of the total available $GABA_A$ receptors. These results suggest that despite the lack of evoked GABA release in SNAP-25 deficient mutants, postsynaptic $GABA_A$ receptors are present and capable of responding to GABA transmission in

SNAP-25 deficient fetal brain and that the decreased amplitude of mPSCs was, therefore, not due to overall decreased $GABA_A$ receptor expression.

To examine whether the decrease in evoked and spontaneous GABAergic activity in *Snap25* null mutants resulted from a comparable deficit of transmitter specific synaptic vesicles, we performed western blot analysis to obtain a quantitative comparison of the relative levels of vesicular glutamate and GABA transport proteins within the synapse. Synaptosome-containing LP2 fractions prepared from the cortex and hippocampus of individual fetal (E17.5) brains were therefore probed for VGAT, VGLUT1, and, as a control, the common protein constituent of synaptic vesicles, synaptophysin. As shown in Figure 2.3A, the intensity of the signals for VGAT and VGLUT1 transporters was comparable between the LP2 fraction of *Snap25* null mutant mice and control extracts. After normalizing the intensities of VGAT and VGLUT1 to synaptophysin in the individual samples, no significant difference was found for the relative signal of VGAT or VGLUT1 between mutant and control LP2 fractions ($p>0.46$; one way ANOVA), indicating that the expression of GABA transporters is not specifically affected in SNAP-25 knockout mutant synapses (Fig. 2.3B). Taken together, these findings suggest that the absence of detectable evoked GABAergic transmission, as well as the 6-fold decrease in the amplitude of GABAergic sPSCs, in fetal SNAP-25 deficient cortex likely result from the inability to form AP-dependent neuroexocytotic machinery rather than the depletion of GABA-

containing synaptic vesicles or a differential expression or absence of vesicular GABA transport proteins.

The VGAT antibody used in these western blots detected a higher molecular weight species that has been reported to reflect a phosphorylated form of the transporter (Bedet et al., 2000). Compared to controls, there was a 55% reduction of this immunoreactive protein species (asterisk, Fig. 2.3A left panel). While phosphorylation of VGAT does not appear to affect the vesicular packaging of GABA, this modification may be involved in the trafficking of vesicles and/or the turnover of the transporter protein (Bedet et al., 2000). This suggests that a decrease in expression of phospho-VGAT in mutant GABAergic synapses could reflect changes in the availability of the readily releasable pool of GABAergic vesicles in SNAP-25 deficient mutants.

Stimulus-evoked vesicular recycling is blocked in both glutamatergic and GABAergic synapses of SNAP-25 deficient neurons.

Because differences in amplitude and frequency of mPSCs between mutant and control brains could reflect the vesicular content or release of transmitter rather than synaptic vesicle fusion per se, we examined neuroexocytosis more directly by measuring vesicular recycling using FM 1-43 uptake (Brumback et al., 2004). Affects on stimulus-driven endocytotic uptake of the styryl dye would provide

additional evidence that the absence of AP-dependent release in SNAP-25 deficient neurons resulted from the loss of evoked neuroexocytosis and not due to fusion of empty vesicles. For these experiments, we used the aldehyde fixable analogue FM 1-43FX to stain dispersed neuronal cultures and assess synaptic vesicle recycling at individual glutamatergic and GABAergic synapses that were subsequently identified by immunocytochemistry with antibodies to the respective vesicular transporters, VGLUT1 and VGAT. While three isoforms of the vesicular glutamate transporter have been identified, only VGLUT1 and VGLUT2 are expressed exclusively in glutamatergic neurons throughout development (Herzog et al., 2001). Although VGLUT2 expression occurs at high levels in glutamatergic neurons during the first week after birth, VGLUT1 that is expressed initially at low levels in the first week postnatally becomes the predominant form in the postnatal brain (Nakamura et al., 2005). In contrast, VGLUT3 is expressed transiently during development in neurons with various neurotransmitter phenotypes (Gras et al., 2005). Therefore, we selected an antibody raised against VGLUT1 that does not cross-react with the two other isoforms, but allowed us to consistently track glutamatergic cells beyond DIV 9-21 for these and the following experiments described below.

Hippocampal neurons derived from *Snap25*$^{-/-}$ mutant and wild type E17.5 fetuses were loaded either by depolarization through application of 90 mM KCl (90 sec) or by exposure to hypertonic sucrose (30 sec) to induce calcium-independent

exocytosis of the readily releasable pool of synaptic vesicles (Rosenmund and Stevens, 1996). After washing away excess dye, neurons were fixed with 4% paraformaldehyde and immunostained with antibodies to either VGLUT1 or VGAT to assess the specific uptake in GABAergic and glutamatergic neurons. As shown in Figure 2.4, wild type cultures showed robust punctate FM dye fluorescent staining after depolarization that colocalized with the punctate staining pattern for VGLUT1 (panel A, panels B1-3) and VGAT (panel D, panels F1-3). In contrast, no FM dye uptake was detectable in SNAP-25 knockout neurons at either glutamatergic or GABAergic immunolabeled synapses (panels C and G, panels D1-3, and H1-3, respectively). Similar images were obtained for wild type and mutant neurons after hypertonic sucrose (not shown). Quantitation of the fluorescence intensity of FM 1-43 dye that colocalized to VGLUT1 and VGAT positive terminals in response to either depolarization (panel I) or hypertonic sucrose (panel J) confirmed that both glutamatergic and GABAergic synapses in wild type neurons readily endocytosed FM1-43 dye, which could be effectively unloaded following a second round of depolarization triggered exocytosis (e.g. destain in panels I and J). Importantly, neither VGLUT1 nor VGAT containing synapses of SNAP-25 deficient neurons showed significant uptake above background fluorescence after high K+ or sucrose, demonstrating a complete lack of stimulus driven-endocytosis and therefore highly compromised neuroexocytosis from VGAT, as well as VGLUT1 containing synapses. Taken together, these results suggest that the lack of evoked PSCs and the decrease in mPSC amplitude seen in electrophysiology recordings

reflects a defect in vesicular fusion in *Snap25*$^{-/-}$ neurons, and is not due to an alternative mechanism, such as the recycling of transmitter depleted synaptic vesicles.

SNAP-25 is localized to the presynaptic terminals of cultured fetal GABAergic neurons

To confirm that SNAP-25 is localized in the presynaptic terminal of fetal GABAergic neurons, we performed immunocytochemical analysis of dispersed neuronal cultures to compare the expression of SNAP-25 in GABAergic and glutamatergic synapses. As described in Materials and Methods, the monocolonal antibody SMI 81 that recognizes an epitope within the N-terminal 31 residues of SNAP-25 (Washbourne et al., 2002), is highly specific and shows no detectable staining in SNAP-25 knockout neurons (Fig. 2.1). Transmitter-specific presynaptic terminals and synaptic contacts were identified using antibodies to the GABA synthetic enzyme GAD 65/67 (Glutamic Acid Decarboxylase) and to the GABA and glutamate vesicular transport proteins as described above. To visualize the presynaptic localization of these transmitter phenotype specific markers with SNAP-25, we used hippocampal neurons prepared from E17.5 mouse fetuses and grown as mixed neuronal and glial cell cultures for 9-21 days. These cultures develop extensive processes and sufficiently fine networks to resolve well defined immunoreactive punctate staining of GABAergic and glutamatergic synapses.

GABAergic neurons were first identified by dual immunocytochemical staining with antibodies to the GABA-specific GAD 65/67 and SNAP-25. GABAergic neurons express GAD in two different isoforms, encoded by separate genes. These two isoforms are expressed in varying proportions, which prompted the use of a rabbit polyclonal antibody raised against a rat fusion protein that recognizes common determinants between the two GAD proteins (Solimena et al., 1993). As shown in Figure 2.5A-B3, confocal images of fluorescent immunostaining with the SNAP-25 monoclonal antibody SMI81 was distributed throughout neuronal processes, but also showed punctate staining consistent with the localization of SNAP-25 in presynaptic terminals. In these cultures, immunostaining for GAD65/67 overlapped extensively with some of these SNAP-25 positive processes. Specifically colocalization between the punctate staining for GAD65/67 and SNAP-25 was revealed, suggesting their expression within the same synapse (see arrows and inset in merged image).

To confirm that the observed punctate staining pattern of SNAP-25 represented presynaptic terminals in GABAergic neurons, we further stained neuronal cultures with antibodies to SNAP-25 and to the vesicular transporters VGAT and VGLUT1, as described above, to distinguish GABAergic and glutamatergic presynaptic terminals, respectively. As with GAD 65/67, immunostaining for the two transporters, VGAT and VGLUT1, resulted in a punctate pattern, consistent with synaptic localization of these vesicular proteins (Fig. 2.5C-F3). By

comparison SNAP-25 staining in many fibers appeared to be less punctate and more continuous with neurites which is likely to represent plasma membrane association of this t-SNARE in fasciculating axons as well as its accumulation in presynaptic terminals (Garcia et al., 1995). Importantly, however, the immunoreactive punctate staining for either transporter coincided with focal immunoreactivity for SNAP-25 (arrows, and see digitally magnified inset), indicating that SNAP-25 is expressed in both terminals with GABA and glutamate containing vesicles. As shown in Figure 2.5I, there was no difference in the extent of colocalization between these two transporters with SNAP-25, suggesting that the SNARE protein expression occurs comparably in GABAergic and glutamatergic terminals. Interestingly, the level of colocalization for VGAT and SNAP-25 immunoreactivity remained remarkably constant throughout 21 days of culture, indicating a persistent expression of this SNARE protein by GABAergic neurons.

Although these results indicated that SNAP-25 expression occurs in both glutamatergic and GABAergic fetal hippocampal presynaptic terminals, a recent study has shown that in neonatal brainstem GABAergic/glycinergic synapses transiently express glutamate transporters and are capable of eliciting glutamatergic transmission (Gillespie et al., 2005). While perhaps specific to certain developmental transitions in neuronal circuitry, such a combined neurotransmitter phenotype in hippocampal cells might complicate any

assignment of neurotransmitter phenotype to SNAP-25 expressing neurons. Therefore, to confirm that cultured hippocampal neurons did not simultaneously express both VGLUT1 and VGAT within the same terminal, we immunostained with antibodies to both transporters. In contrast to the colocalization seen with either of the two transporters and SNAP-25, the punctate pattern obtained for VGAT and VGLUT1 dual-staining revealed little or no colocalization of the transporters themselves, even within fasciculated bundles of both GABAergic and glutamatergic fibers (Fig. 2.5G, H, quantitated in panel I). This suggests that GABAergic and glutamatergic synapses are distinct in these cultured hippocampal neurons, and that few if any terminals contain substantial amounts of both GABAergic and glutamatergic vesicles. Quantitating the pixel overlap of the images confirmed that there was minimal (<5%) colocalization of the two vesicular transporters. This non-overlapping pattern was found even at the earliest time point analyzed (DIV 9). Overall, the colocalization of the transporters with SNAP-25, but not between each other, indicate that these two distinct neurotransmitter phenotypes are expressed in cultured hippocampal neurons and that SNAP-25 is present in the presynaptic terminals of both developing GABAergic and glutamatergic neurons.

Mature GABAergic neurons express SNAP-25

The previous results demonstrated that SNAP-25 expression is required for synaptic transmission in fetal GABAergic neurons. We carried out further

evaluation of SNAP-25 expression by mature GABAergic neurons by employing immunocytochemistry and fluorescent in situ hybridization (FISH) to examine protein and mRNA expression in adult mouse brain. We examined SNAP-25 accumulation in synaptic terminals of GABAergic and glutamatergic neurons in adult mice, by performing fluorescent immunohistochemistry with the same SNAP-25, VGAT, and VGLUT1 antibody combinations used above for colocalization studies in cultured neurons. In coronal sections of hippocampus, we observed marked differences in the distribution of glutamatergic and GABAergic synapses, with VGLUT1 immunoreactivity found primarily in the stratum oriens and stratum radiatum layers and VGAT staining mainly localized to the stratum pyramidale layer. Despite the overall anatomical segregation of these terminals, VGAT and VGLUT1 positive staining was interspersed at the borders of these hippocampal layers, consistent with the intermingling of excitatory and inhibitory synapses. Quantification of the pixel overlap of the punctate immunostaining for the transporters with SNAP-25 detected with the mAb SMI81 showed comparable colocalization between SNAP-25 and VGLUT1 (53%) or for SNAP-25 and VGAT (47%) (Fig. 2.6A-D, G). In contrast, little or no pixel colocalization was found after co-staining for VGLUT1 and VGAT (Fig. 2.6E-G), consistent with separate and distinct GABAergic and glutamatergic synaptic terminals. To further assess the colocalization of SNAP-25 with punctate staining that could represent glutamatergic or GABAergic synaptic boutons; we selected a minimal size for pixel clusters of the immunostainning for the vesicular transporters before evaluating their colocalization with SNAP-25.

This indicated that ~95% of clustered pixels representing either VGAT- or VGLUT1-positive terminals overlapped with SNAP-25 reactivity (see Supplemental Figure 2.1).

As shown in Figure 2.7A-B, immunofluorescent staining revealed a rich abundance of GABAergic synapses within the ventral posteriolateral (VPL) nucleus of the thalamus, which likely represents afferent projections from the reticular nucleus. Within this region, SNAP-25 immunoreactivity was also widespread, and its colocalization with VGAT was consistent and robust throughout. In addition, punctate staining for VGLUT1 found in the VPL also overlapped with SNAP-25 immunoreactivity, consistent with colocalization of SNAP-25 with these interspersed glutamatergic terminals (Fig. 2.7C-D). Overlapping punctate staining reflecting colocalized expression of VGAT/SNAP-25 was also observed throughout the cortex and caudate putamen (data not shown). As seen in immunostaining of fetal neuronal cultures, the punctate colocalized staining of VGAT and VGLUT1 with SNAP-25, coupled with the non-overlapping pattern of the two transporters, indicated that SNAP-25 is translocated to presynaptic terminals of both glutamatergic and GABAergic neurons in the adult CNS.

Because comparable levels of pixel overlap were found in both glutamatergic and GABAergic synapses, we used FISH analysis to examine the colocalized expression of SNAP-25 and GAD 65/67 mRNAs. FISH analysis of multiple,

differently labeled probes provides a complimentary method with increased resolution and clarity as it allows for cell-by-cell comparison and avoids the ambiguity found when probing for protein expression in overlapping and interspersed synaptic connections. Therefore, we used a double-label FITC and Cy3 amplification (Guzowski et al., 1999; Vazdarjanova et al., 2002; Vazdarjanova and Guzowski, 2004) of fluorescein-labeled GAD65/67 and digoxigenin-labeled SNAP-25 antisense riboprobes to identify cells co-expressing both mRNAs in four separate anatomical regions. For these experiments, a mixture of both GAD65 and GAD67 fluoroscein-labeled riboprobes were transcribed separately and equal amounts of the two probes were pooled before hybridization. As shown in Figure 2.8A, GAD65/67 was readily detected in neurons dispersed throughout layers I-V of cortex; consistent with the distribution of GABAergic interneurons and the prominent hybridization for SNAP-25 mRNA within these neurons. In hippocampus, a similar colocalization of double-labeled GAD65/67 and SNAP-25-positive neurons was found with GABAergic neurons throughout the stratum oriens, stratum pyramidale, and stratum radiatum of the CA1-CA3 regions (Fig. 2.8B). As shown in Figure 2.8C, a striking pattern was also observed in thalamus where GAD65/67-positive neurons that are distinctly partitioned within the thalamic reticular nucleus also exhibited robust expression of SNAP-25 mRNA (Fig. 2.8C). In contrast, throughout neighboring regions, such as the ventral posteriolateral (VPL) nucleus and the internal capsule, SNAP-25 mRNA was clearly detected in the absence of GAD65/67 hybridization, consistent with the production of this t-SNARE in glutamatergic neurons.

Similarly, neurons within the caudate putamen hybridized with both SNAP-25 and GAD65/67 probes (Fig. 2.8D). Quantitative analysis of the FISH images revealed that virtually all GAD65/67 positive neurons were also SNAP-25 positive throughout all four brain regions (Fig. 2.8E).

2.5 Discussion

SNAP-25 is a critical component of the neural SNARE complex that contributes to the calcium-triggered exocytosis of neurotransmitter by facilitating membrane fusion between synaptic vesicles and the presynaptic plasma membrane. Numerous studies have implicated the involvement of SNAP-25 and neural SNARE complex in the release of a wide range of neurotransmitters. Here, we provide electrophysiological evidence from SNAP-25 deficient mutant and wild type mice that extends the repertoire of this t-SNARE to a role in GABAergic transmission. Moreover, we observed SNAP-25 localization in the presynaptic terminals of GABAergic neurons throughout maturation, inferring that GABAergic function is dependent on SNARE-mediated neurotransmission throughout development.

Interestingly, in contrast to the evidence presented here, it has been reported recently that maturation of GABAergic neurons may be accompanied by a decrease in detectable SNAP-25 expression and an increase in resistance of GABA transmission and vesicular cycling to BoNT/A and BoNT/E. These observations have been interpreted to indicate that SNAP-25 does not participate in the SNARE complexes responsible for GABAergic transmission (Verderio et al., 2004; Frassoni et al., 2005). The activity of Clostridium neurotoxins in blocking transmitter release, however, varies greatly depending on the serotype of toxin, as well as the susceptibility of different neurons to the surface binding and activation of the holotoxin (Purkiss et al., 2001; for review, see Montecucco

et al., 2004). The spatial orientation, grouping, and organization of specialized gangliosides within a lipid raft have been shown to alter the plasma membrane binding affinity of botulinum neurotoxins (Yowler et al., 2002). Differential vulnerability to toxins, therefore, can depend on the regulated expression of gangliosides that act as neuron-specific, toxin co-receptors (Vyas et al., 2001). For example, while BoNT/A effectively binds to and blocks transmitter release at peripheral cholinergic synapses (Black and Dolly, 1987), there is considerable variability in the binding and uptake of neurotoxins into neurons across different anatomical regions in the adult rat brain (Williams et al., 1983; Black and Dolly, 1987). Thus, the inability to detect SNAP-25 mediated GABA release, when assayed by neurotoxin sensitivity, could be due to changes in cell surface receptors during the maturation of inhibitory neurons, which result in decreased binding and increased resistance to BoNT/A and BoNT/E intoxication. Use of *Snap25* null mutants in which the expression of this SNARE protein is uniformly ablated in all neurons, therefore, circumvents possible variability in toxin efficacy. Nevertheless, because the mutants do not survive birth, functional studies of integrated neuronal connectivity are limited to the developing fetal brain.

As Frassoni et al. (2005), we observed SNAP-25 immunoreactivity in cultured GABAergic neurons after 9-10 days in culture. However, in contrast to their observation of decreasing coexpression in GAD positive neurons over several days in culture, we found no change in the extent of colocalization of SNAP-25

with immunoreactive GAD 65/67 and VGAT (or of comparable levels of VGLUT1 for glutamatergic neurons) in hippocampal neurons cultured for up to 21 days. Similarly, we show extensive co-expression of SNAP-25 with GAD65/67 and VGAT, which define GABAergic neurons, within several anatomically distinct regions of the adult mouse brain. Based on these results, we propose that GABAergic neurons maintain SNAP-25 expression for stimulus-driven GABA release throughout synaptic maturation.

Although all inhibitory neurons that we examined expressed SNAP-25 mRNA, there were differences in the level of hybridization distinguished by fluorescent intensity among different subpopulations of GABAergic neurons, consistent with the differential expression of this t-SNARE across brain regions (Oyler et al., 1989). Interestingly, regional differences have also been detected in the expression of SNAP-25 isoforms, generated through alternative splicing (Bark et al., 1995; Boschert et al., 1996). This indicates that the expression of SNAP-25 may be coupled with distinct physiological properties of neurotransmission in different neurons. The two SNAP-25 isoforms have been shown to significantly affect the recruitment of catecholaminergic vesicles into the readily releasable pool of chromaffin cells (Sorensen et al., 2003). Further studies will examine the possibility that regulated expression of SNAP-25 and its isoforms may confer differences that suit the differing requirements of transmission by various GABAergic interneurons and projection neurons.

The absence of stimulus-evoked GABA postsynaptic responses in SNAP-25 deficient fetal brain indicates the role of SNAP-25-containing SNARE complexes mediating AP-dependent GABAergic synaptic transmission. This is further supported by FM 1-43 dye uptake experiments that demonstrated the lack of evoked vesicular recycling at both glutamatergic and GABAergic terminals in SNAP-25 knockout mutant neuronal cultures. Moreover, recent ongoing studies have shown that the expression of SNAP-25 transgenes is able to rescue and restore both evoked GABAergic and glutamatergic transmission to *Snap25* null mutant neurons in culture (I. D. Martinez and J. B. Sorensen, personal communication). The observation of spontaneous GABAergic mPSC activity is, however, also consistent with the AP-independent release of other transmitters previously seen in this mutant (Washbourne et al., 2002). The amplitude and frequency of these spontaneous events, however, appears to vary considerably between transmitter systems. For example, although both the frequency and amplitude of GABAergic mPSCs recorded in mutant cortex were significantly reduced when compared to controls, cholinergic miniature endplate potentials at mutant neuromuscular junctions were found to have increased frequency and amplitude (Washbourne et al., 2002). This contrasts with the comparable glutamatergic mEPSCs recorded from cortex of mutant and control fetal brain, and the similar level of the slow, non Ca^{2+}-triggered component of catecholamine secretion of mutant chromaffin cells that is thought to represent the slow release from an unprimed pool of secretory granules (Sorensen et al., 2003). Interestingly, mEPSCs recorded from cultured hippocampal neurons occur at

significantly lower frequency, but maintain the same amplitude as control mEPSCs This does suggest, however, that while quantal release of transmitter does occur in the absence of SNAP-25, presumably promoted by other SNARE complexes, changes in pre- and postsynaptic properties of these spontaneous events can be influenced by the loss of evoked transmission.

The reason for the decreased frequency of mPSCs in both mutant GABAergic and mutant glutamatergic neurons is not clear. It has been shown that in fetal *Snap25* null mutants, calbindin- and calretinin-positive populations of GABAergic neurons are generated and migrate appropriately within the cortex (Molnar et al., 2002). However, the possibility remains that mutant neurons may exhibit alterations in activity-dependent synaptic refinement resulting in less efficient formation of functional synapses, thereby causing a decreased mPSC frequency.

The decreased amplitude of GABAergic mPSCs in SNAP-25 deficient brain slices was not accompanied by a corresponding decrease in either VGAT expression or a decrease in the overall GABAA receptor responsivity. In fact, application of exogenous GABA elicited a markedly higher level of receptor activation in mutant slices as compared to controls, suggesting up-regulation of GABAA receptors in response to the lack of evoked GABAergic transmission. The reduced amplitude recorded from these spontaneous events nevertheless may reflect deficits in filling, trafficking and/or targeting of GABA-containing

synaptic vesicles. For example, the amplitude of mIPSCs can be dependent on the efficient uptake and conversion of extracellular glutamate to GABA (Mathews and Diamond, 2003). The abolition of evoked glutamatergic transmission in *Snap25* null mutants could lead to decreased availability of glutamate within the synaptic cleft, thus diminishing the uptake of this amino acid by GABAergic neurons. Impaired glutamatergic release may therefore depress GABA synthesis and contribute to a decreased quantal content of GABAergic vesicles. In addition, reuptake of GABA appears to be regulated by the interaction of syntaxin 1a with the GABA transporter GAT1 (Wang et al., 2003). In the absence of SNAP-25, syntaxin 1a that is no longer recruited into SNARE complexes may be available to interact and down-regulate GAT1, thereby leading to decreased GABA reuptake and repackaging into GABAergic vesicles. Decreased levels of phospho-VGAT, in addition to the decreased mPSC frequency discussed above, may further reflect alterations in the trafficking and turnover of a pool of GABA-containing vesicles that are compromised in SNAP-25 deficient terminals.

Identifying proteins that mediate the release of different neurotransmitters provides a means to differentiate between those proteins that are central to the basic mechanisms of neuroexocytosis from those that are modulatory and shape the physiological characteristics of transmitter-specific synapses. Demonstration of the expression of SNAP-25 in GABA-containing terminals and the dependence on this SNARE protein for GABAergic transmission therefore supports an

essential role for SNAP-25 in a universal neural SNARE complex necessary for calcium-triggered release within diverse neurotransmitter systems. However, although SNAP-25 is a key element for evoked transmitter release, the inclusion of its developmentally-regulated isoforms into neural SNARE complexes also affects the release properties of neurosecretion (Sorensen et al., 2003; Nagy et al., 2005) and may participate in short-term plasticity and maturation of excitatory synaptic transmission (Bark et al., 2004). This suggests that SNAP-25 can serve a pivotal role, both as a fundamental constituent and as a modulatory component of the presynaptic membrane fusion machinery underlying neurotransmission.

2.6 Figure Legends

Figure 2.1. SMI 81 monoclonal antibody shows robust and specific immunoreactivity in both cultured neurons and western blots.

A, Wild type E17.5 cultured hippocampal neurons immunostained at 12 days in vitro (DIV) using SMI 81 monoclonal antibody and viewed by confocal microscopy with a 63x objective (n.a.=1.4; optical slice 0.81 µm). Probing for SNAP-25 (green; Alexa 488) resulted in staining that was both continuous throughout the neurites with regular punctate accumulations, but not appreciably within the soma or around the nucleus (blue; ToPro3). B, SMI 81 showed no apparent immunoreactivity in *Snap25* null mutant neuronal cultures. C. Protein fractions prepared from the cortices of *Snap25*$^{-/-}$ and wild type animals at E17.5 (mutant, lane 1; wild type, lane 2; 30 µg) and PN24 (wild type, lane 3; 1.0 µg) fractionated on a 12% SDS-PAGE gel, blotted, and probed for SNAP-25. SMI 81 immunoreactivity was evident in wild type lanes as a single band at approximately 25 kDa with no cross reactivity in the mutant lane. The synaptic vesicle protein synaptophysin was used as a loading control.

Figure 2.2. GABAergic transmission could not be evoked in SNAP-25 deficient mutant neurons, although spontaneous currents persist.

A, Whole-cell patch clamp recordings of field-stimulation evoked postsynaptic currents (PSCs) obtained from cortical slices of E17.5 control and *Snap25* null mutant fetuses. GABAergic responses were isolated by recording in the presence of NBQX (10μM) + APV (100μM) to block glutamatergic transmission. Representative tracings are presented on the left with respective calibrations. The trace (dotted) obtained after application of 20 μM bicuculline to control slices is superimposed over the recording in the absence of $GABA_A$ receptor inhibition and indicates GABAergic origin of the response. Mutant slices did not show any detectable response to stimulation. B, Recordings of spontaneous PSCs (sPSCs) in the absence of TTX. C, sPSCs from *Snap25* null mutant slices (n=9) were decreased in frequency (26.1 fold) and amplitude (6.0 fold) compared to controls (n=5; $p<0.001$). D, Miniature PSCs (mPSC) recorded in the presence of TTX (0.5 μM). E, Both the amplitude (5.2 fold) and frequency (12.9 fold) of TTX-resistant mPSCs were decreased in *Snap25* null mutants (n=3) compared to controls (n=3; $p<0.001$). TTX treatment, however, did not significantly reduce the frequency or amplitude of mPSCs recorded from SNAP-25 deficient slices (see text) indicating that action potential-dependent responses that contribute to the sPSCs of control slices are completely absent in *Snap25* null mutants. F, Response of *Snap25* null mutant (n=5) and control slices (n=5) to bath application of GABA (50 μM). Note that the response to exogenous GABA in *Snap25* null mutant slices was more robust than control (8.4 fold, $p<0.001$, n=8)

suggesting that the decreased amplitude for mIPSCs recorded from mutant slices was not due to inherent receptor defects and that $GABA_A$ receptors may be up-regulated in SNAP-25 deficient fetal brain.

Figure 2.3. Similar levels of synaptic vesicle proteins expressed in SNAP-25 deficient and control mice.

A, Crude synaptic vesicle fraction (LP2; 2.5 µg) prepared from cortex and hippocampus of E17.5 *Snap25*$^{-/-}$ and control littermates were fractionated on a 10% SDS-PAGE gel, blotted and probed with antibodies to synaptophysin and VGAT, or VGLUT1, as indicated. B, Three animals per genotype were assayed in duplicate with the mean of each animal's repeated values being normalized to the average synaptophysin levels of the control group. One-way ANOVA analysis showed no difference between levels of immunoreactive VGAT, VGLUT1 and synaptophysin in mutant and control fractions, indicating that neither the total vesicular content nor specific GABA or glutamate containing vesicles are significantly decreased in SNAP-25 deficient neurons. The slower migrating species (indicated by an asterisk) recognized by VGAT antibodies likely reflects phosphorylated VGAT (see Results).

Figure 2.4: Vesicular recycling within both glutamatergic and GABAergic terminals requires SNAP-25.

Hippocampal neurons prepared from both *Snap25*$^{-/-}$ (KO) and control (WT) E17.5 mice grown for 12 days in culture (DIV12) were loaded with in the presence of 15 μM FM 1-43FX by application of either high K^+ or hypertonic sucrose buffer as described in the Material and Methods; control wild type neurons were also destained by applying a subsequent 90 sec exposure to 90 mM K^+ to demonstrate exocytotic release and washout of the endocytosed FM dye. After fixation, and immunostaining with either VGLUT1 or VGAT to distinguish dye uptake at glutamatergic or GABAergic synapses, the neurons were viewed by laser confocal microscopy. Representative confocal fluorescent images are shown in panels A-H, with panels A, C, E, G on the left depicting merged images of FM 1-43 dye fluorescence and transporter immunostaining taken at 63X (optical slice 0.81 μm; scale bar 50 μm). The panels on the right (B1-3, D1-3, F1-3, H1-3) are series of separated color and merged images of the areas outlined in the low power images (white box) that were digitally magnified 7X (Adobe Photoshop; scale bar 7 μm). Insets in the far right merged panels are further digitally enlarged images of the puncta indicated by white arrowheads. Note that FM1-43 dye (green in all panels) is taken up readily by wild type neurons with equal and consistent colocalization in both glutamatergic (red, B1-3) and GABAergic (red, F1-3), whereas in SNAP-25 deficient terminals there is no detectable FM 1-43 dye uptake regardless of their neurotransmitter phenotype (VGLUT, red, D1-3, VGAT, red H1-3), consistent with of the lack of stimulus-

evoked vesicular recycling in these mutant neurons. Panel I shows the quantitative data of FM 1-43 dye intensity over immunoreactive puncta obtained for each transporter after high K^+ stimulation. Note that levels of FM 1-43 dye fluorescence in loaded wild type terminals were highly significant ($p<0.001$) compared to both destained control synapses and to knockout mutant synapses which did not differ from background fluorescence. Panel J compares the relative amount of FM 1-43 fluorescence between wild type and control neurons in a similar series of experiments using hyperosmotic sucrose to promote exocytosis. As in panel I, although wild type neurons showed robust vesicular recycling, no detectable fluorescence was observed over VGLUT or VGAT immunoreactive puncta of *Snap25*$^{-/-}$ mutant neurons indicating that there was no loading of a readily releasable pool of synaptic vesicles at either glutamatergic or GABAergic terminals in these neurons.

Figure 2.5: Immunostaining of cultured hippocampal neurons reveals SNAP-25 colocalization with GAD65/67, VGAT, and VGLUT1 in presynaptic terminals.

Hippocampal neurons prepared from E17.5 mouse fetuses were cultured, probed with antibodies to the indicated proteins and viewed by confocal microscopy as described in the Materials and Methods. Panels A-H are representative confocal fluorescent images of stained neurons at DIV15. Left panels A, C, E, G are merged images of dual staining taken at 63X (optical slice 0.81 μm; scale bar 50 μm). Panels on the right (B1-3, D1-3, F1-3, and H1-3) are series of separated color and merged images at higher digital magnification of the areas outlined in the low power images (scale bar 20 μm). Insets in the far right merged panels are digitally enlarged images of the puncta indicated by white arrows. Note that dual immunostaining for SNAP-25 (green), and GAD65/67 (red, panels A and B1-3), VGAT (red, panels C, D1-3), and VGLUT1 (red, panels E, F1-3) show marked colocalization (yellow in merged images) of punctate fluorescence for transmitter-specific proteins with SNAP-25, consistent with expression of the SNARE protein in GABAergic and glutamatergic terminals (and see panel I). Arrowheads indicate punctate staining of SNAP-25 that is not colocalized with transmitter-specific antibodies indicating accumulation of SNAP-25 outside terminals of the indicated transmitter phenotype. Panels G and H, dual staining for transporters VGLUT1 (green) and VGAT (red) show little or no colocalization. Panel I summarizes quantitative data of colocalized punctate staining from dual-stained cultures at DIV9, 12, 15, and 21 obtained using Metamorph software (***, $p<0.001$).

Figure 2.6. SNAP-25 immunoreactivity colocalizes with VGAT in the CA1 pyramidal layer of the adult hippocampus.

Thirty micron coronal sections of adult mice (>60 days old) were co-immunostained with antibodies to SNAP-25 and either vesicular transporters VGAT or VGLUT1, and as a control for specificity co-immunostained with anti-VGLUT1 and VGAT antibodies. After nuclear counterstaining with ToPro3 (blue), the sections were imaged by laser confocal microscopy as described in the Materials and Methods. Right panels (A, C, and E) are images taken using a 63X objective (optical slice 0.81 μm), and the series of panels on the left (B1-3, D1-3, and F1-3) show separate channel and merged images of the boxed areas with overlapping red and green pixels depicted as yellow after digital magnification to 420x. A, B, SNAP-25 (green) colocalizes with VGAT (red) within the stratum pyramidale (S.P.) layer of the CA1 region. C, D, VGLUT1 immunoreactivity (red) localized to the stratum oriens (S.O.) and stratum radiatum (S. R.), also colocalizes with SNAP-25 (green). E, F Non-overlapping immunoreactivity for VGLUT1 (green) and VGAT (red) seen throughout the hippocampus. Note that although the patterns of immunostaining for the two transporters are predominantly located in different layers of the hippocampus (panel E), in regions where VGAT and VGLUT1 immunoreactive puncta are interspersed, such as the border of the stratum oriens and pyramidale (boxed area in panel E, magnified in panels F), there is virtually no colocalization of these transporters. G, VGLUT1 and VGAT colocalize to a similar extent with SNAP-25. The proportion of pixel overlap in representative immunofluorescent images for transporters VGLUT1

and VGAT with SNAP-25 (VGLUT1/SNAP-25 and VGAT/SNAP-25, respectively) and between the two transporters (VGLUT1 and VGAT) was quantified using Metamorph software. The histogram represents average values (error bars, SEM) obtained from 12 images taken from 3 animals (***, $p<0.001$). Scale bar: A, C, E 20 μm; B, D, F 3.0 μm.

Figure 2.7. Colocalization of SNAP-25 and VGAT immunoreactivity occurs in the thalamus.

Confocal images of immunofluorescent staining for SNAP-25 (green), VGAT(red), and VGLUT1 (red) in 30 μm sections of PN>60 wild type mice were obtained as described in Materials and Methods. A, B1-4, SNAP-25 and VGAT immunostaining within the ventral posteriolateral (VPL) of the thalamus. The separate color channel and merged images illustrate the extensively overlapping punctate pattern of robust immunoreactivity observed for VGAT and SNAP-25 (arrows) in this region; consistent co-compartmentalization of SNAP-25 and the GABA transporter within presynaptic terminals of GABAergic neurons. The arrowhead indicates an example of colocalized punctate stained structure after 5x digital enlargement in far upper right corner. C, D1-4, In contrast, VGLUT1 staining was scarce within the VPL, although small regions with prominent immunoreactivity were evident. Immunofluorescent staining for the glutamate transporter within these patches also appeared punctate and overlapped with SNAP-25 immunostaining, again reflecting the expression of SNAP-25 in glutamatergic presynaptic terminals. A, C 20x magnification, scale bar = 50 μm; B, D 63x magnification, scale bar = 20 μm.

Figure 2.8. Adult GABAergic neurons express both SNAP-25 and GAD65/67 mRNA in several brain regions.

Fluorescent in situ hybridization of SNAP-25 and GAD65/67 cRNA probes, followed by nuclear counterstaining with DAPI (blue), was performed on 30 μm coronal brain sections of PN>60 wild type mice as described in the Materials and Methods. Sections were visualized using a wide field fluorescent microscope and imaged with Metamorph software. A-D, Fluorescent images of hybridization for SNAP-25 (red) and GAD65/67 (green), and ToPro3 staining in selected brain regions in separate color channels, and a color merged micrograph (far right). A, primary motor cortex (layers I-V); B, hippocampal CA1 region (SO, stratums oriens; SP, stratums pyramidale; SR, stratums radiatum, and SL, lacunosum molecular), C, thalamus (VPL, ventral posteriolateral nucleus, RT, reticular nucleus, and ic, internal capsule), and D, caudate putamen. GAD65/67 positive nuclei throughout these selected anatomical regions display robust expression and colocalization with SNAP-25 mRNA (white arrows, digitally enlarged in white box in upper far right corner of the merged image). Images were taken with a 20x objective; scale bar for A-D: 50 μm. E, Quantitation of colocalized GAD65/67 and SNAP-25 hybridization with 20x magnification. The fraction of GAD65/67-positive cells with overlapping SNAP-25 hybridization, determined using ImageJ software, demonstrates that nearly all cells expressing GAD65/67 mRNA co-expressed SNAP-25 mRNA in each brain region assayed. Statistical analysis was carried out by one-way ANOVA with Bonferroni post hoc comparisons.

Supplemental Figure 2.1: SNAP-25 immunoreactivity found in both GABAergic and glutamatergic puncta within the hippocampus.

A, B, Using data acquired from dual stain immunofluorescence of adult hippocampal slices from Figure 2.6, SNAP-25, VGAT, and VGLUT1 staining patterns were reanalyzed to visually assign a pixel intensity threshold that isolated focal accumulations defined as puncta (left panel, red signal) from other immunofluorescently labeled structures of the tissue (left panel, gray signal). The area of a representative single pixel clusters (12 pixels x 12 pixels or 144 pixels2; left panel, white circle) was selected to define the lower size limit of immunofluorescent puncta. To quantify the colocalization of the transporters with SNAP-25, these criteria were assigned for both VGAT and VGLUT1 immunostaining (left panel, A and B, respectively). Those puncta that overlapped with SNAP-25 (green, middle panels) were subtracted using NIH ImageJ analysis software. The very few clusters representing either VGAT or VGLUT1 stain that did not colocalize with SNAP-25 (right panel, white arrows). C, Statistical analysis revealed that SNAP-25 is found in ~95% of either VGAT- (black bar) or VGLUT1-positive puncta (gray bar). In contrast, analysis of the puncta resulting from VGAT and VGLUT1 dual staining showed little, if any, colocalization (white bar). Sections were imaged by laser confocal microscopy as described in the Materials and Methods using a 63X objective (optical slice 0.81 μm) and with digital magnification to 420X. The histogram represents average values (error bars, SEM) obtained from nine images taken from three animals (***, $p<0.001$).

2.7 Figures

Figure 2.1

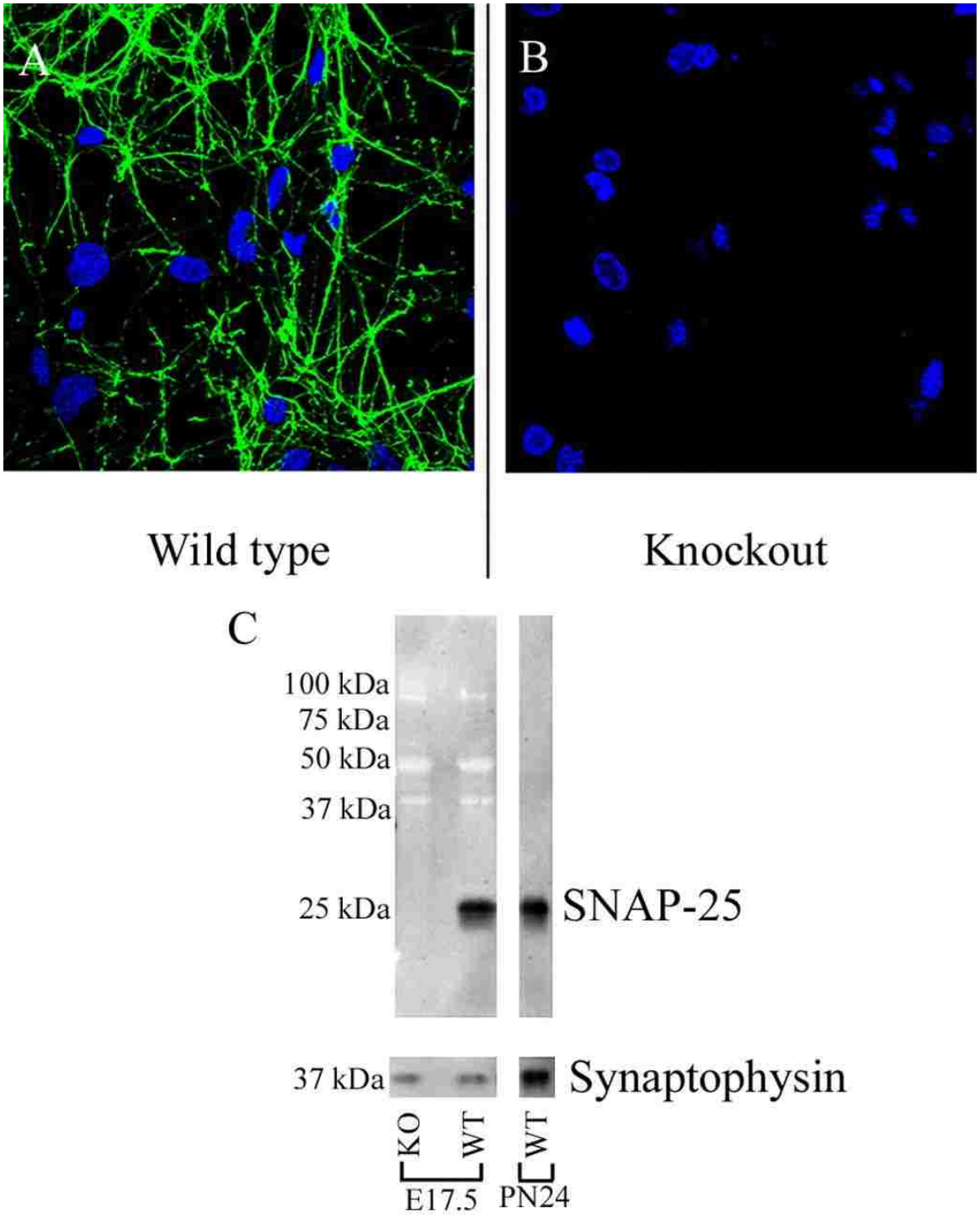
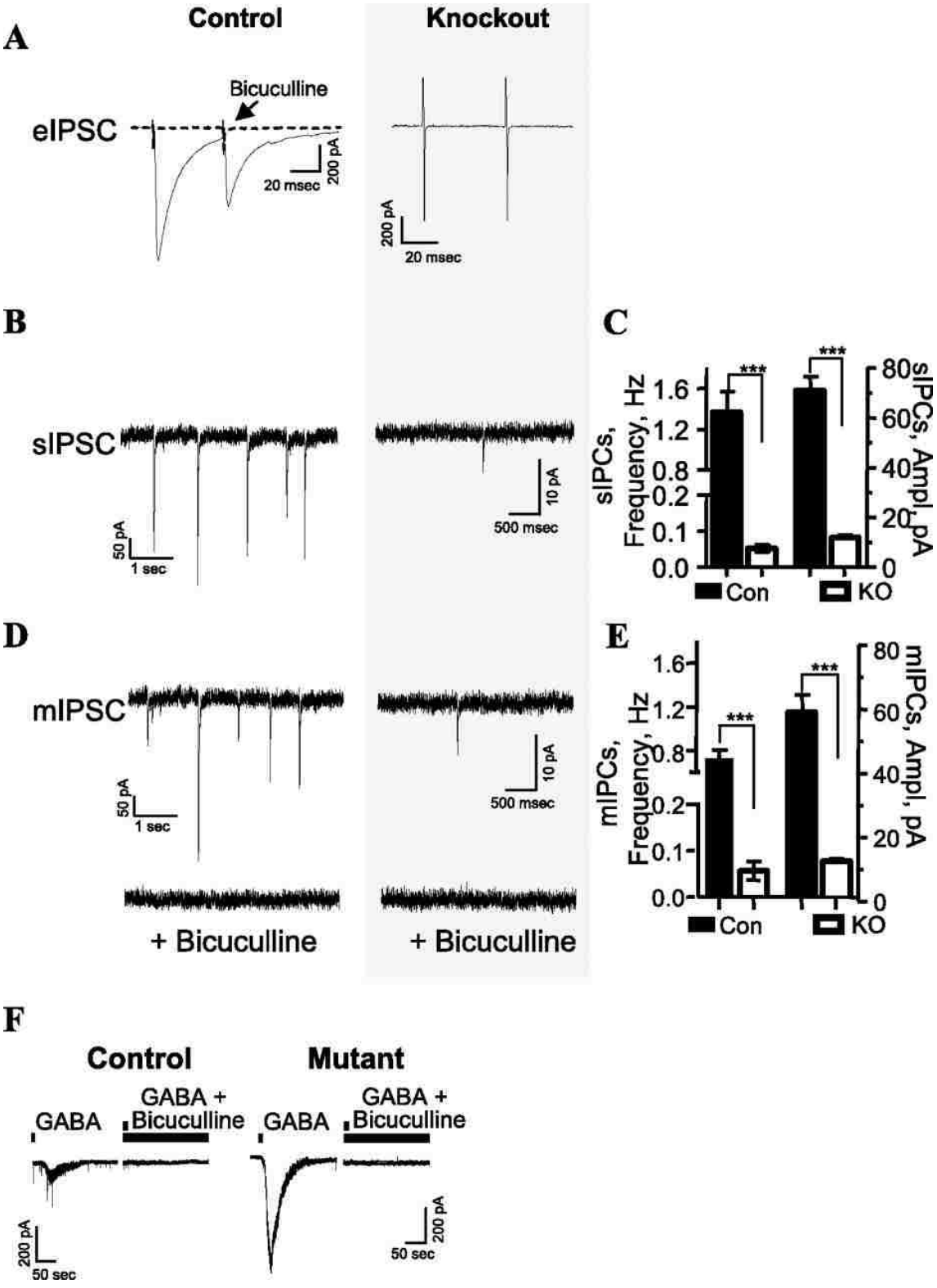


Figure 2.2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Bicuculline
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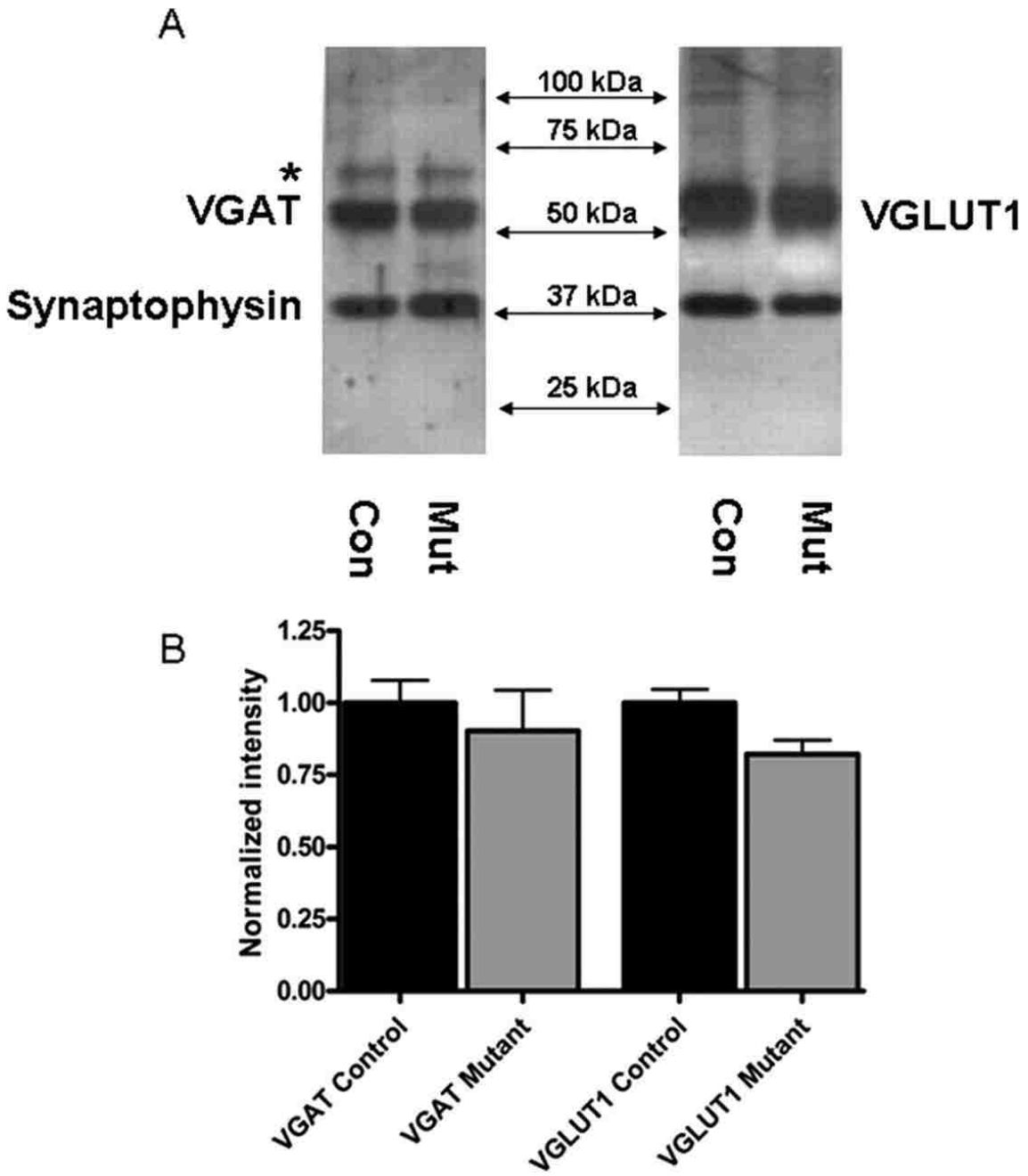
20 msec
200 pA
20 msec
B
sIPSC
50 pA
1 sec
10 pA
500 msec
C
***
***
sIPCs, Frequency, Hz
1.6
1.2
0.8
0.2
0.1
0.0
80
60
40
20
0
sIPCs, Ampl, pA
Con
KO
D
mIPSC
50 pA
1 sec
10 pA
500 msec
+ Bicuculline
+ Bicuculline
E
***
***
mIPCs, Frequency, Hz
1.6
1.2
0.8
0.2
0.1
0.0
80
60
40
20
0
mIPCs, Ampl, pA
Con
KO
F
Control
Mutant
GABA
GABA + Bicuculline
GABA
GABA + Bicuculline
200 pA
50 sec
200 pA
50 sec

Figure 2.3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100 kDa
75 kDa
*
VGAT
50 kDa
VGLUT1
Synaptophysin
37 kDa
25 kDa
Con
Mut
Con
Mut
B
Normalized intensity
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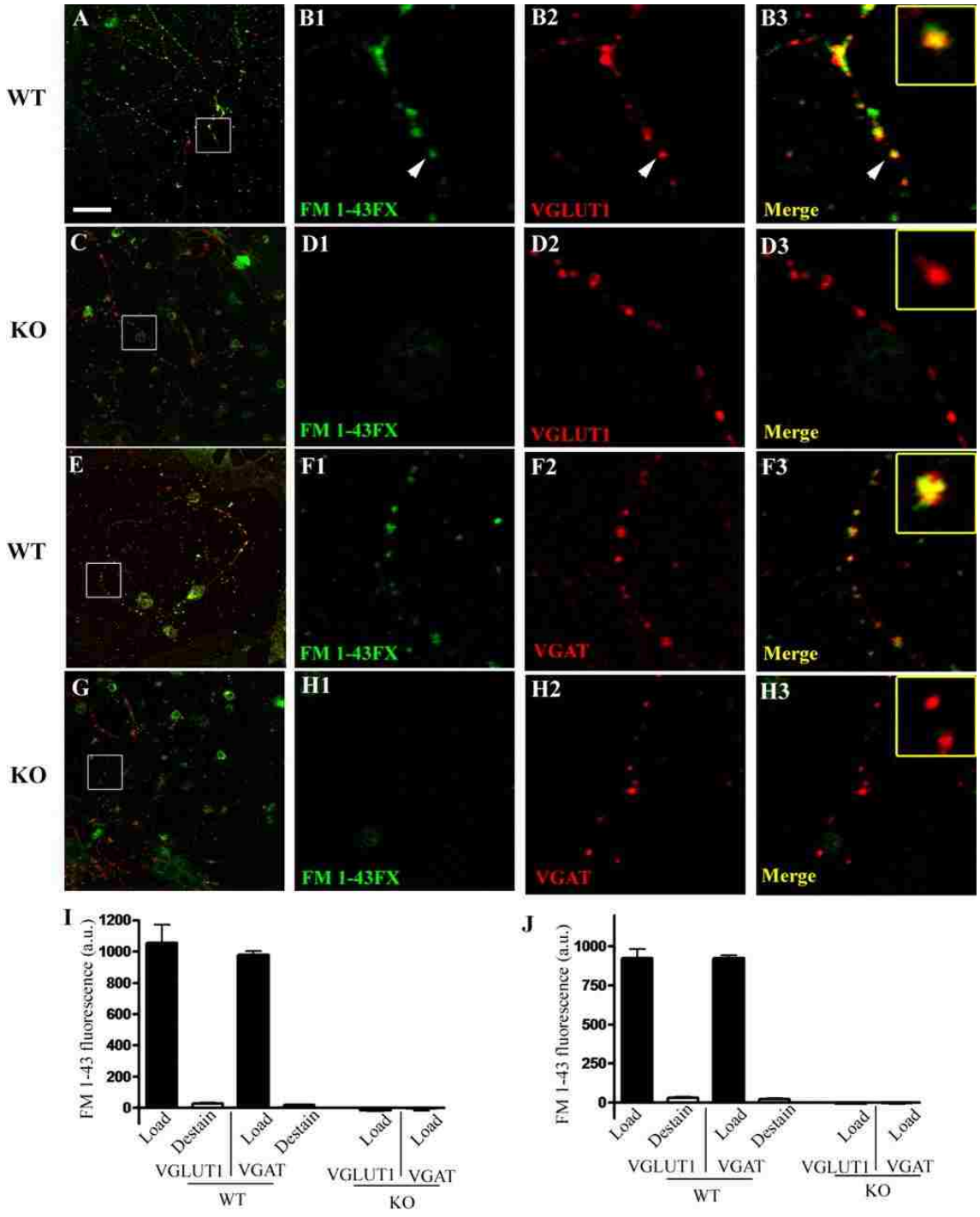
1.00
0.75
0.50
0.25
0.00
VGAT Control
VGAT Mutant
VGLUT1 Control
VGLUT1 Mutant

Figure 2.4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B2
B3
WT
FM 1-43FX
VGLUT1
Merge
C
D1
D2
D3
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VGLUT1
Merge
E
F1
F2
F3
WT
FM 1-43FX
VGAT
Merge
G
H1
H2
H3
KO
FM 1-43FX
VGAT
Merge
I
FM 1-43 fluorescence (a.u.)
1200
1000
800
600
400
200
0
Load
Destain
Load
Destain
Load
Load
VGLUT1
VGAT
VGLUT1
VGAT
WT
KO
J
FM 1-43 fluorescence (a.u.)
1000
750
500
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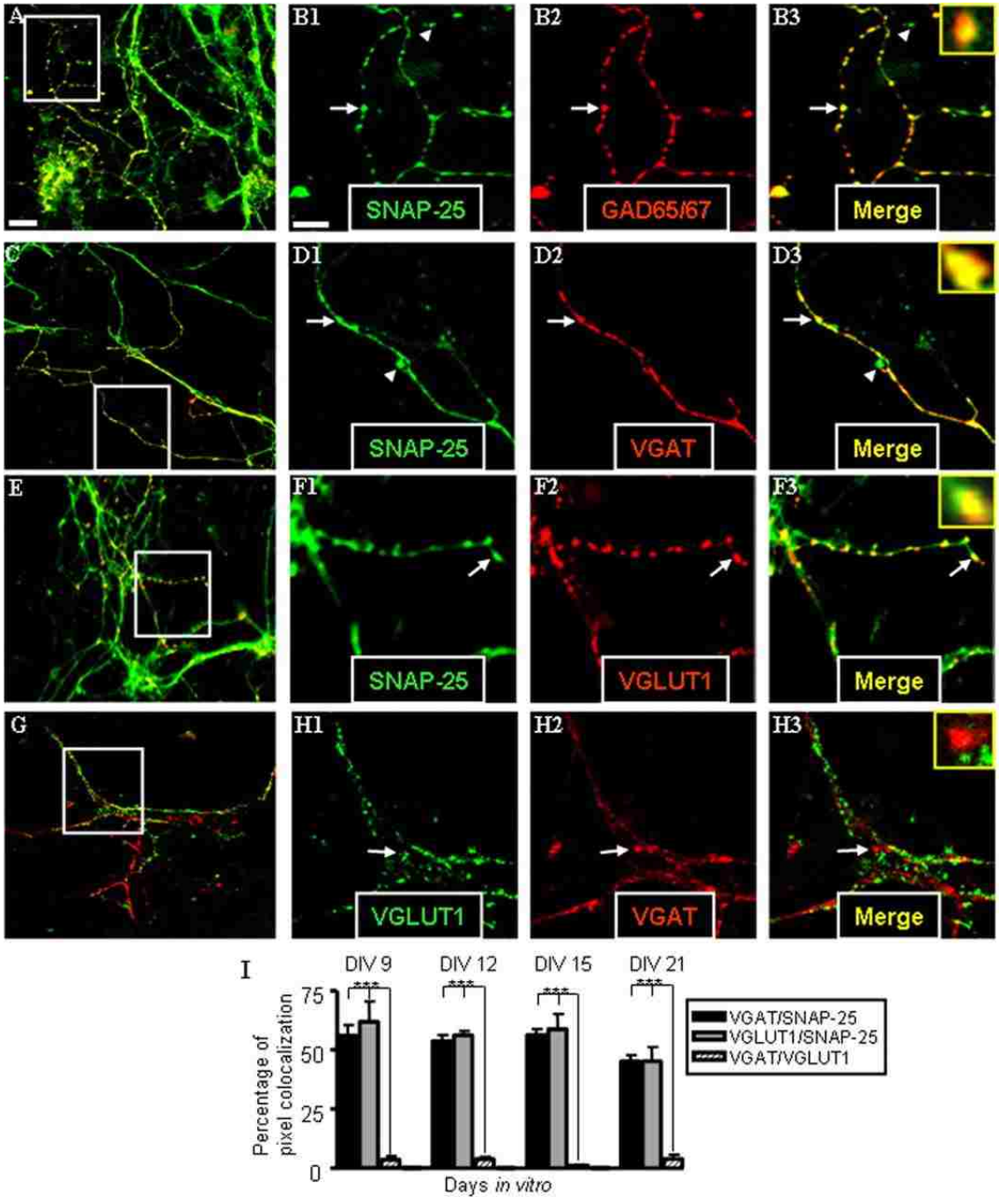
VGAT
WT
KO

Figure 2.5


A
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SNAP-25
B2
GAD65/67
B3
Merge
C
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SNAP-25
D2
VGAT
D3
Merge
E
F1
SNAP-25
F2
VGLUT1
F3
Merge
G
H1
VGLUT1
H2
VGAT
H3
Merge
I
DIV 9
DIV 12
DIV 15
DIV 21
Percentage of pixel colocalization
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50
25
0
Days in vitro
VGAT/SNAP-25
VGLUT1/SNAP-25
VGAT/VGLUT1

Figure 2.6

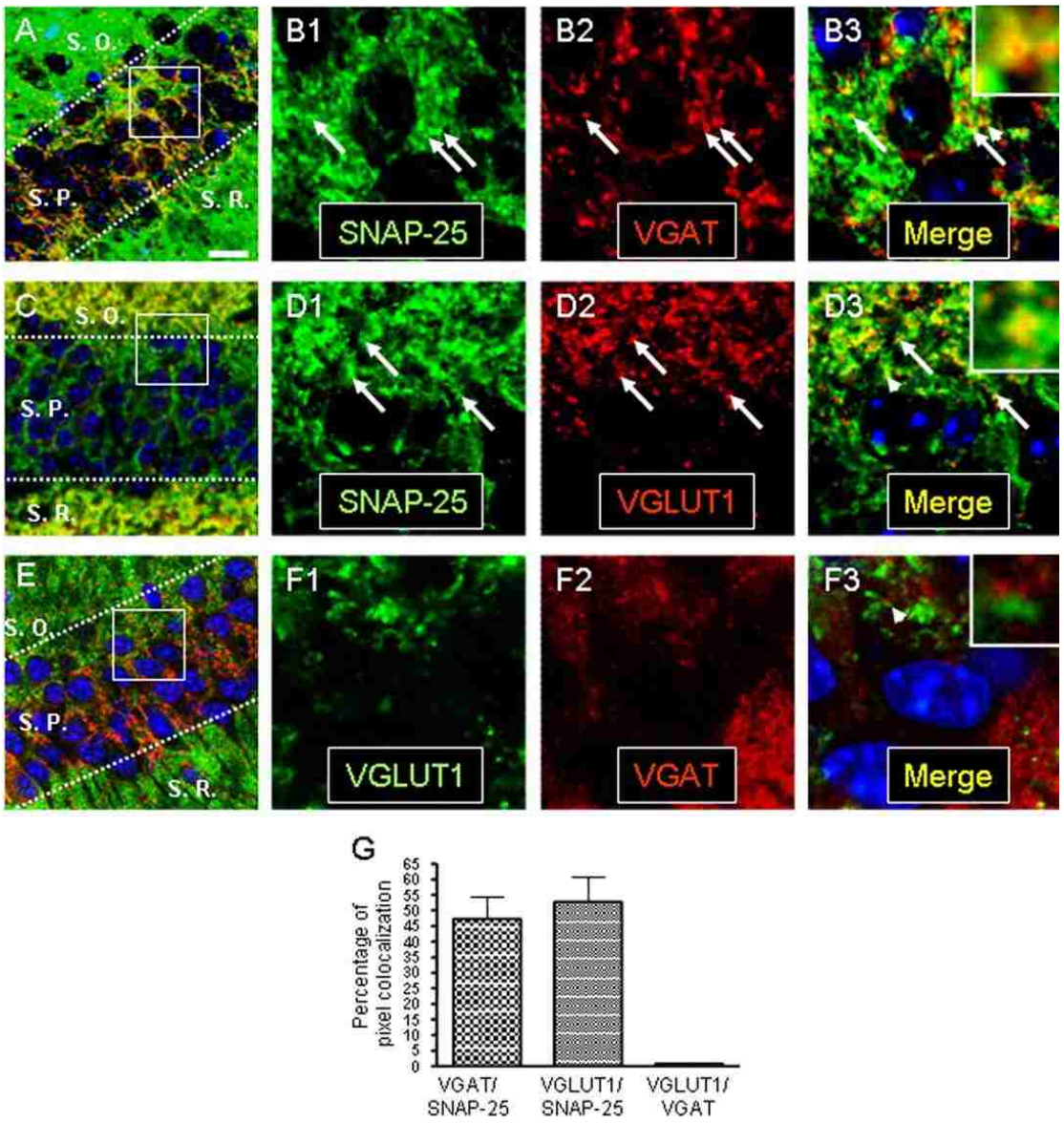

A
S. O.
S. P.
S. R.
B1
SNAP-25
B2
VGAT
B3
Merge
C
S. O.
S. P.
S. R.
D1
SNAP-25
D2
VGLUT1
D3
Merge
E
S. O.
S. P.
S. R.
F1
VGLUT1
F2
VGAT
F3
Merge
G
Percentage of pixel colocalization
0
5
10
15
20
25
30
35
40
45
50
55
60
65
VGAT/ SNAP-25
VGLUT1/ SNAP-25
VGLUT1/ VGAT

Figure 2.7

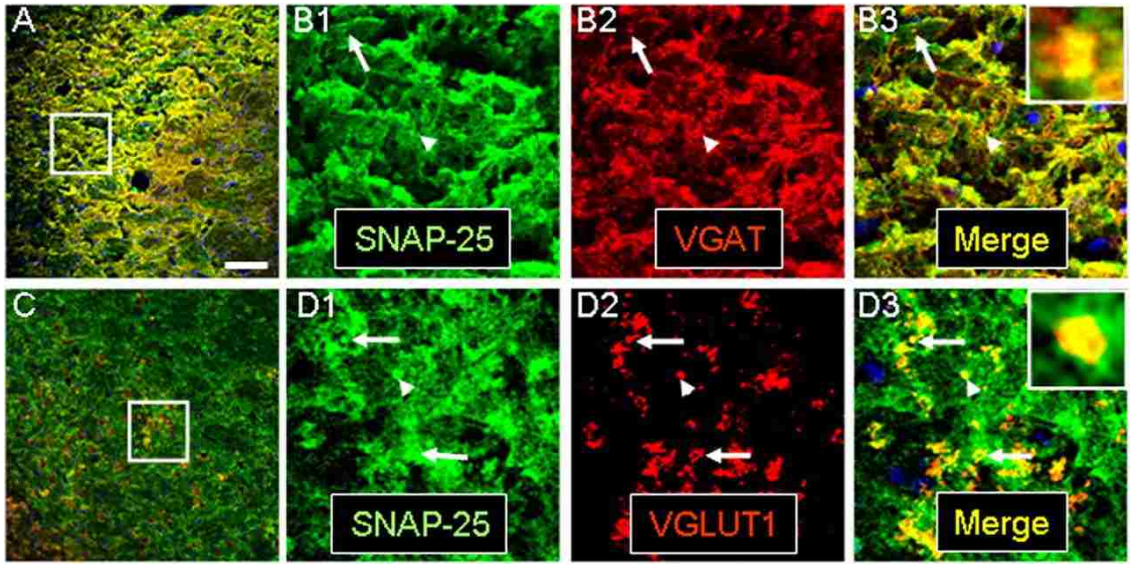
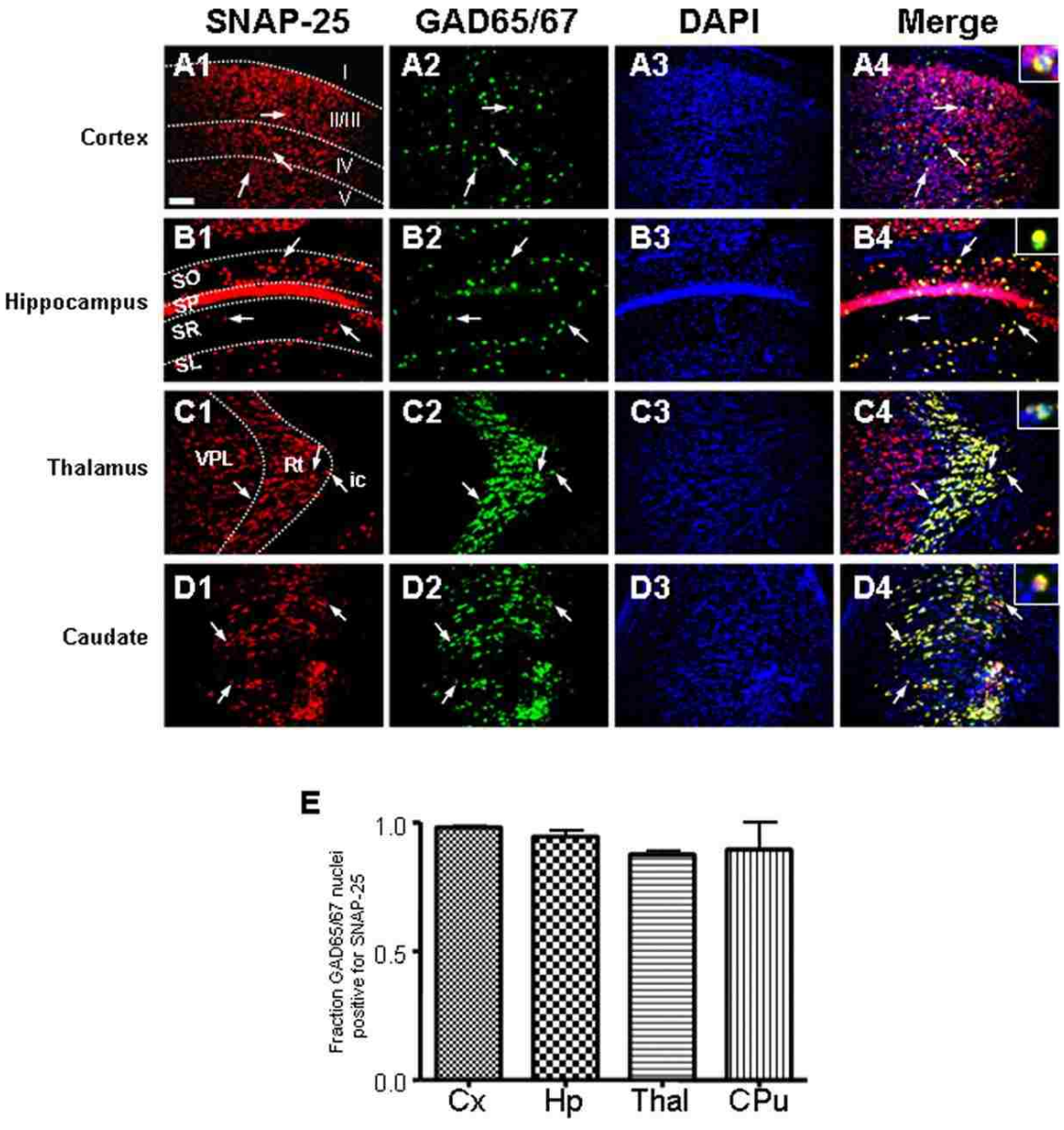


Figure 2.8


SNAP-25
GAD65/67
DAPI
Merge
Cortex
Hippocampus
Thalamus
Caudate
A1
A2
A3
A4
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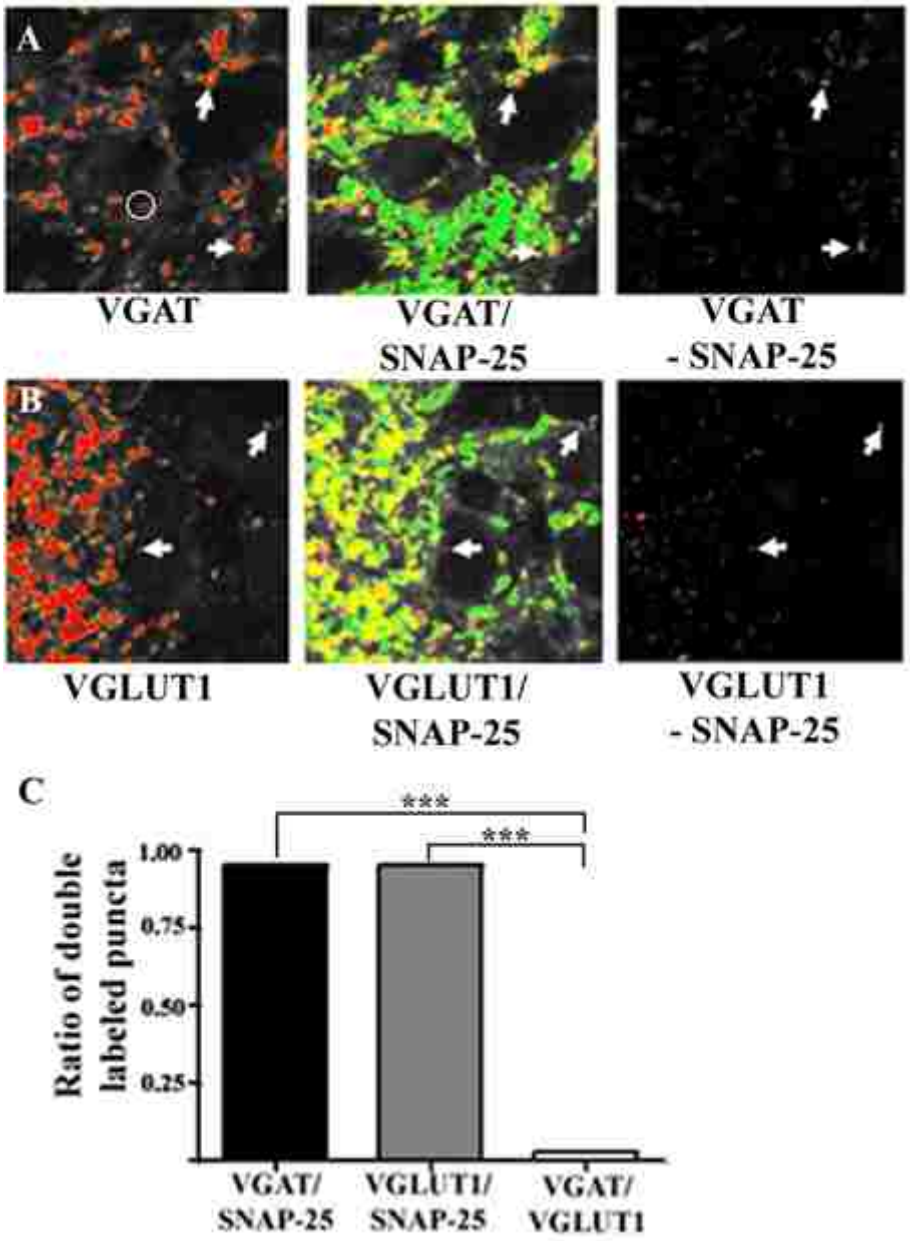
B3
B4
C1
C2
C3
C4
D1
D2
D3
D4
I
II/III
IV
V
SO
SP
SR
SL
VPL
Rt
ic
E
Fraction GAD65/67 nuclei positive for SNAP-25
1.0
0.5
0.0
Cx
Hp
Thal
CPu

Supplemental Figure 2.1



2.8 Acknowledgements:

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3. The Role of the t-SNARE SNAP-25 in Action Potential-Dependent Calcium Signaling and Expression in GABAergic and Glutamatergic Neurons

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3.1 Abstract

Background

The soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex, comprised of SNAP-25, syntaxin 1A, and VAMP-2, has been shown to be responsible for action potential (AP)-dependent, calcium-triggered release of several neurotransmitters. However, this basic fusogenic protein complex may be further specialized to suit the requirements for different neurotransmitter systems, as exemplified by neurons and neuroendocrine cells. In this study, we investigate the effects of SNAP-25 ablation on spontaneous neuronal activity and the expression of functionally distinct isoforms of this t-SNARE in GABAergic and glutamatergic neurons of the adult brain.

Results

We found that neurons cultured from *Snap25* homozygous null mutant (*Snap25*$^{-/-}$) mice failed to develop synchronous network activity seen as spontaneous AP-dependent calcium oscillations and were unable to trigger glial transients following depolarization. Voltage-gated calcium channel (VGCC) mediated calcium transients evoked by depolarization, nevertheless, did not differ between soma of SNAP-25 deficient and control neurons. Furthermore, we observed that although the expression of SNAP-25 RNA transcripts varied among neuronal populations in adult brain, the relative ratio of the transcripts

encoding alternatively spliced SNAP-25 variant isoforms was not different in GABAergic and glutamatergic neurons.

Conclusion

We propose that the SNAP-25b isoform is predominantly expressed by both mature glutamatergic and GABAergic neurons and serves as a fundamental component of SNARE complex used for fast synaptic communication in excitatory and inhibitory circuits required for brain function. Moreover, SNAP-25 is required for neurons to establish AP-evoked synchronous network activity, as measured by calcium transients, whereas the loss of this t-SNARE does not affect voltage-dependent calcium entry.

3.2 Background

Regulated neurotransmission at chemical synapses underlies neural communication and is likely to contribute to complex brain functions, such as synaptic plasticity and memory storage. Neurotransmitter release requires fusion of synaptic vesicles that is mediated by the neuronal SNARE complex at release sites of presynaptic terminals (for review, see Rizo and Sudhof, 2002; Jahn and Scheller, 2006). This core heteromeric protein assembly, comprised of the t-SNAREs syntaxin 1, and SNAP-25 situated at the target or plasma membrane and the v-SNARE VAMP-2/synaptobrevin on secreting vesicles, is responsible for membrane fusion that underlies the Ca^{2+}-triggered neuroexocytosis that is required for AP-dependent neurotransmission signaling point-to-point communication between neurons, as well as the regulated secretion from neuroendocrine cells. Evidence suggests, however, that this neural SNARE complex may not be required for constitutive synaptic activity in the absence of presynaptic depolarization, although deletion of SNARE protein genes does alter characteristics of spontaneous neurotransmitter release events detected by recordings of AP-independent miniature postsynaptic currents or "minis" (mPSCs). For example, the analysis of neurons and neuroendocrine cells of SNAP-25 null mutant mice, generated by homologous recombination-mediated disruption of this t-SNARE gene (Washbourne et al., 2002), has demonstrated the selective abrogation of evoked neurotransmission, leaving constitutive release of neurotransmitter in catecholaminergic (Sorensen et al., 2003), GABAergic (Tafoya et al., 2006), glutamatergic and cholinergic systems

(Washbourne et al., 2002) intact, despite varying effects on the amplitude and frequency of these transmitter-specific release events.

In addition to a well-documented role in membrane fusion for neuroexocytosis and neurotransmitter release, the t-SNAREs SNAP-25 and syntaxin 1 also associate with voltage-gated calcium channels (VGCCs) where they are thought to modulate steady-state inactivation of channel opening thereby regulating calcium currents in response to membrane depolarization (see Catterall 1999 for review). In particular, SNAP-25 has been shown to specifically interact with one isoform of the P/Q-type VGCC (rbA) to limit calcium currents mediated by this channel (Zhong et al., 1999). Acute interference of SNAP-25 expression has been reported to lead to increased depolarization-induced calcium transients in cultured neurons (Verderio et al., 2004). Increased intracellular calcium ($[Ca^{2+}]_i$) would be expected to increase the frequency and amplitude of mPSCs (for review, see Collin et al., 2005), yet previous evidence has shown that genetic deletion or BoNT cleavage of SNAP-25 can result in decreased mPSC frequency (Capogna et al., 1997, Trudeau et al., 1998, Bronk et al., 2007; Delgado-Martinez et al., 2007). This raises the question of whether SNAP-25 plays any role in managing calcium influx through VGCCs.

Synaptogenesis and maturation of functional synaptic connectivity is accompanied by dramatically increased levels of SNAP-25 (Catsicas et al.,

1991), as well as a significant change in the relative expression of transcripts encoding two isoforms of the protein that are produced by alternative splicing between divergent, tandem arranged copies of a single exon (Bark et al., 1995). Interestingly, the representation of these two SNAP-25 isoforms differs markedly between neurons of the mature nervous system and in neurosecretory cells (Bark et al., 1995; Boschert et al. 1996; Jacobsson et al., 1996; Gonelle-Gispert et al., 1999; Grant et al., 1999; Jacobsson et al., 1999; Jacobsson et al., 1999), suggesting that these isoforms are likely to impart physiological distinctions to presynaptic function that are ultimately required for the distinct properties of neurons that make up diverse components of neural circuitry. Consistent with this idea, the isoforms have been reported to promote differences in the recruitment of primed vesicles for neuroexocytosis in both chromaffin cells and hippocampal neurons (Sorensen et al., 2003; Delgado-Martinez et al., 2007), and in hippocampal short-term plasticity (Bark et al., 2004). Nevertheless, whether such fine-tuning due to regulation of the expression of SNAP-25 and its isoforms plays such a role in sculpting properties of synaptic transmission in specific neuronal cell-types, and in particular excitatory glutamatergic or inhibitory GABAergic neurons, has not been fully resolved (Verderio et al., 2004; Frassoni et al., 2005; Tafoya et al., 2006).

To explore this idea further, we investigated the role of SNAP-25 in synaptic communication and on calcium dynamics in neuronal cultures prepared from

either *Snap25*$^{-/-}$ mutant or control mice. We then extended our analysis to examine differences in the expression of SNAP-25 isoforms between glutamatergic and GABAergic neurons during development and within different functional networks. Our results support the idea that regulation of SNAP-25 contributes to the developmental fine-tuning of a universal SNARE complex required for mature, stimulus-evoked synaptic transmission both in cultured neurons and by major populations of excitatory and inhibitory neurons.

3.3 Results

Ca^{2+} signaling in control and SNAP-25 deficient neurons

Previous studies have established that cultured SNAP-25 deficient hippocampal neurons fail to trigger depolarization-dependent presynaptic vesicular endocytosis or to evoke postsynaptic currents (Washbourne et al., 2002; Tafoya et al., 2006, Bronk et al., 2007). To complement these investigations, we performed live-cell imaging of intracellular Ca^{2+}dynamics in mixed cultures of hippocampal neurons and glial cells that were prepared from wild type *Snap25*$^{+/+}$, heterozygous *Snap25*$^{+/-}$, and homozygous *Snap25*$^{-/-}$ fetuses. Other studies have shown that when cultured rodent CNS neurons adopt a synchronous pattern of synaptic network activity, it is reflected by oscillating Ca^{2+} transients that can be monitored in the soma using the calcium indicator Fura 2 (Murphy et al., 1992; Lawrie et al., 1993; Nunez et al., 1996; Bacci et al., 1999). Ratiometric (350/380 nm) measurements were taken over neuronal and glial cell bodies (5–9 cells per coverslip) that were identified retrospectively by both morphology and a sustained Ca^{2+} response to depolarization after bath application of media containing high K^+ (Fig. 1, and see Methods for details).

Neurons of both wild type and heterozygous cultures exhibited characteristic synchronous oscillating Ca^{2+} transients that had an overall frequency of 5.4 transients/min (range 1.4–8/min) (Fig. 1A). The amplitudes of oscillations produced by *Snap25*$^{+/+}$ and *Snap25*$^{+/-}$ neurons were virtually identical (0.229 ±

0.018 versus 0.229 ± 0.025 ΔFura-2 ratio; n = 7 cultures of each genotype, 5 cells counted per culture, Fig. 2A), indicating that a reduced level of SNAP-25 in *Snap25*$^{+/-}$ neurons did not affect the calcium currents measured from soma under these conditions. As expected, addition of tetrodotoxin (TTX, 1 μM) abolished neuronal Ca^{2+} oscillations (Fig. 1A), demonstrating the dependence of these events on the propagation of action potentials and presumably synaptic release of neurotransmitters between cultured neurons. By comparison, as shown in Fig. 1B, cultures obtained from *Snap25*$^{-/-}$ fetuses showed virtually no spontaneous Ca^{2+} oscillatory activity (n = 8 cultures) in neurons. These SNAP-25 deficient neurons, however, did exhibit a robust Ca^{2+} response after K^{+} depolarization (Fig. 1C), consistent with the presence of functional VGCCs despite the lack of Ca^{2+}-triggered evoked neurotransmitter release in the absence of neural SNARE proteins (Schoch et al., 2001; Washbourne et al., 2002).

In *Snap25*$^{+/+}$ and *Snap25*$^{+/-}$ cultures the resting $[Ca^{2+}]_i$ level in neurons after the TTX block was significantly lower than that measured at the trough between oscillations prior to TTX exposure (Fura 2 ratio 0.861 ± 0.044, no treatment; 0.805 ± 0.031, TTX; n = 10 cultures, $p < 0.008$). However, in contrast to control cultures, TTX did not significantly affect resting $[Ca^{2+}]_i$ in *Snap25*$^{-/-}$ neurons (Fura-2 ratio 0.852 ± 0.023, no treatment, compared to 0.845 ± 0.021 with TTX; n = 7, $p > 0.3$).

To examine whether the mechanisms that underlie the generation of Ca^{2+} transients were intact in the *Snap25*$^{-/-}$ neurons, we compared their response with the Ca^{2+} rise exhibited by control neurons after K^+-induced depolarization. Bath application of 55 mM K^+ in the presence of TTX resulted in robust increases in $[Ca^{2+}]_i$ levels in null mutant neurons that were not significantly different from control wild type or heterozygote neurons (Fig. 1C, quantified in Fig. 2B). Pre-incubation with selective voltage gated calcium channel blockers, moreover, did not show an overall effect on the relative contribution of L-, N- and P/Q-type channels as distinguished by the progressive addition of nimodipine (10 μM) and conotoxins GIVA and MVIIC (Fig. 2C). These results suggest that while SNAP-25 deficient neurons do not undergo Ca^{2+} oscillatory behavior, presumably due to the absence of SNARE medicated evoked release, the general machinery required for Ca^{2+} responses in the soma of these neurons has not been significantly altered.

Astrocyte Ca^{2+} dynamics

We observed spontaneous Ca^{2+} transients in astrocytes in the mixed cultures prepared from *Snap25*$^{+/+}$, *Snap25*$^{+/-}$ and *Snap25*$^{-/-}$ mutant animals. As expected for astrocyte signals, Ca^{2+} transients in these cells were not synchronized between individual astrocytes, and were not prevented by TTX. However, when high K^+ was applied to *Snap25*$^{+/+}$ and *Snap25*$^{+/-}$ cultures in the presence of TTX, most preparations showed a clear increase in the frequency and amplitude of

astrocyte Ca^{2+} oscillations (Fig. 1D, grouped data shown in Fig. 3), that occurred in parallel with sustained Ca^{2+} elevations in neurons. Astrocyte Ca^{2+} signals were prominent in *Snap25*$^{-/-}$ cultures and the characteristics of spontaneous events appeared similar to those observed in *Snap25*$^{+/+}$ and *Snap25*$^{+/-}$ cultures, and were not prevented by TTX. However, in contrast to *Snap25*$^{+/+}$ and *Snap25*$^{+/-}$ cultures, the average frequency of spontaneous astrocyte Ca^{2+} oscillations significantly decreased (rather than increased) during high K^+ exposures (Fig 3A), although no significant change in the amplitude of the events was detected (Fig. 3B). This suggests that a lack of transmitter release from depolarized neurons impairs neuron-glia communication in these cultures.

Developmental regulation of SNAP-25 in GABAergic neurons

While it has been demonstrated that SNAP-25 is expressed and required for stimulus-driven synaptic transmission by both glutamatergic and GABAergic neurons in fetal mouse brain and in culture (Washbourne et al., 2002; Tafoya et al., 2006; Bronk et al., 2007; Delgado-Martinez et al., 2007), it has been also proposed that differential expression of this t-SNARE may lead to differences in calcium dynamics between inhibitory and excitatory synapses (Verderio et al., 2004) and thereby possibly contribute to the physiological diversity observed between these neurons (for review, see Jonas et al., 2004). However, in the preceding experiments we did not detect a significant effect of SNAP-25 expression on the modulation of calcium responses in cultured neurons.

Neuronal cultures do not exhibit the appropriate synaptic circuitry that is developed in the intact brain. We considered therefore whether the developmentally regulated isoforms of SNAP-25 (Bark and Wilson, 1994; Bark et al., 1995) might be responsible, in part, for the distinctive synaptic properties of glutamatergic and GABAergic neurons. For example, while there is a general shift in the relative levels of the two isoforms during brain maturation, the expression of the earlier expressed SNAP-25a persists in most neuroendocrine cells, as well as in certain discrete neuronal populations (Boschert et al., 1996; Jacobsson et al., 1996; Gonelle-Gispert et al., 1999; Grant et al., 1999; Jacobsson et al., 1999; Jacobsson et al., 1999; Bark et al., 2005). Furthermore, the expression of the variant SNAP-25 isoforms has been shown to affect the size of the RRP in hippocampal neurons (Delgado-Martinez et al., 2007), as well as in adrenal chromaffin cells (Sorensen et al., 2003; Nagy et al., 2005), and has been suggested to contribute to developmental changes in hippocampal short-term synaptic plasticity (Bark et al., 2004).

To examine whether two SNAP-25 isoforms are differentially expressed in GABAergic neurons and therefore might play a role in tailoring the distinct properties of synaptic activity in these neurons, we utilized GAD67-GFP (Δneo) transgenic mice that bear a knockin insertion of green fluorescent protein (eGFP) coding sequence at the glutamate decarboxylase 67 (GAD67) gene locus (Tamamaki et al., 2003). Expression of this fluorescent marker by virtually all

GABAergic populations enabled us to distinguish and isolate GABAergic from GFP-negative, non-GABAergic and largely excitatory, neuronal populations. The level of expression of the isoforms was determined by real-time quantitative RT-PCR (qRT-PCR) using SNAP-25a and 25b transcript-specific primers (Grant et al., 1999). Preliminary experiments established that the primer sets were equally efficient in amplifying the specific isoform sequences from cDNA templates, and RNA transcripts prepared from brains of SNAP-25a overexpressing mutant and control mice, as well as from cells transfected with cDNAs encoding the individual isoforms (see Methods), thereby validating the RT-PCR assay as a measure of the relative expression of the two isoform transcripts.

A global description of isoform expression in the mature brain was first obtained by using fluorescence-activated cell sorting (FACS) to select eGFP-positive cells from freshly dissociated cerebral cortices of adult mice (Fig. 4). As expected, initial experiments demonstrated that a distinct GFP-positive (GFP_{pos}) population of cells could be readily distinguished and isolated from GAD67-GFP (Δneo), but not control wild type littermates (compare Fig. 4 panels A and B). Subsequent qRT-PCR analyses were then carried out on the two fractions of sorted cells from transgenic animals to obtain GFP-negative (GFP_{neg}, non-GABAergic cells) and GFP_{pos} (GABAergic) populations (Fig. 4A, black and green arrow, respectively). The purity of the sorted cell populations was confirmed by qRT-PCR analysis for mRNA transcripts encoding the transmitterspecific transporters, VGLUT1 and

VGAT. Our results, summarized in Table 1, demonstrate that a relatively high level of VGAT transcripts was detected in the absence of a VGLUT1 signal in GFP_{pos} samples, and conversely, a similarly high level of expression of VGLUT1 was determined for GFP_{neg} cells in which VGAT RNA was undetectable. Because the GFP_{neg} cell population was not positively selected for any marker, it is likely composed of non-GABAergic neurons, the vast majority being glutamatergic, as well as astrocytes and other glial cell types. However, since SNAP-23, but not SNAP-25, is expressed in astrocytes (Parpura et al., 1995; Montana et al., 2004) and little, if any SNAP-25 can be detected in oligodendrocytes (Madison et al., 1999), the amplification of SNAP-25 isoforms is primarily, if not exclusively, from glutamatergic neurons; a premise that is supported by the relatively high abundance of VGLUT1 transcripts detected by qRT-PCR.

As shown in Fig. 4C, qRT-PCR analysis of the GFP-expressing GABAergic cortical neurons obtained from adult mice showed nine-fold greater expression of SNAP-25b compared to SNAP-25a transcripts, indicating 90% of the total SNAP-25 mRNA population was composed of SNAP-25b and 10% of SNAP-25a coding mRNAs. Virtually identical results were obtained from the GFP_{neg} cell fraction, consistent with previous findings based on an RNase protection assay of total adult brain RNA (Bark et al., 1995).

Since the relative expression of the SNAP-25 isoforms is dynamically regulated in cortex and other brain regions during development (Bark et al., 1995; Boschert et al., 1996), we next investigated the relative abundance of the specific transcripts in GFP_{pos} and GFP_{neg} populations of cortical neurons prepared from P7 mice. As shown in Fig. 4D, in contrast to the greater abundance of SNAP-25b transcripts in adult brain, the level of the transcripts encoding the two isoforms was equivalent in both GABAergic and non-GABAergic populations of neonatal cortical cells. These results indicate GABAergic neurons in the developing and mature neocortex, principally represented by interneurons, express the same relative levels of SNAP-25 isoforms as the majority of cortical excitatory neurons that predominantly express SNAP-25b in the adult brain.

Relative expression of SNAP-25 isoforms does not vary in different anatomical regions

The previous experiment examined the regulation of SNAP-25 isoforms within a fraction of cortical cells based globally on neurotransmitter phenotype. However, this analysis did not address whether the differential expression of the isoforms is common between different populations of GABAergic neurons. Therefore in order to evaluate the expression of the two isoforms within specific GABAergic and glutamatergic neuronal populations, we used laser capture microdissection (LCM) to isolate small groups of GABAergic and glutamatergic neurons from functionally distinct areas within the adult brain of GAD67-GFP (Δneo) transgenic

mice. Using a fixation procedure optimized for LCM/qRT-PCR analysis (see Methods), eGFP-expressing cells in several representative anatomical regions were readily identified and single-cell microdissection allowed capture of these selected neurons without apparent excision of neighboring cells (Fig. 5A). To obtain a broad sampling of GABAergic populations, we collected pools of approximately 50 cells including projection neurons from the reticular nucleus of the thalamus, caudate, cerebellar Purkinje cells, as well as hippocampal interneurons located in both the stratum oriens and radiata of the CA1 region. In addition, we harvested non-GFP expressing glutamatergic neurons, specifically CA1 hippocampal pyramidal neurons and cerebellar granule cells, based on their distinct cellular morphology and location in well-defined areas of the brain. As before, we confirmed the purity of the selected GABAergic and glutamatergic neuron samples using qRTPCR analysis of VGLUT1/VGAT expression (Table 1).

Analysis of cellular transcripts by qRT-PCR revealed no significant difference in the relative levels of SNAP-25a and SNAP-25b transcripts between GABAergic projection neurons and interneurons, regardless of anatomical location, and nonGABAergic (glutaminergic) hippocampal pyramidal and cerebellar granule cells ($p = 0.25$, one-way ANOVA, Tukey's Multiple Comparison Test, Fig. 5B). Overall, the predominance of SNAP-25b transcripts amongst these adult brain regions selected by LCM (88–93% of the total) was consistent with the isoform transcript levels obtained for adult cortical cells isolated by FACS (Fig. 4C),

extending these findings to neuronal populations in the hippocampus, cerebellum and the subcortical regions of the thalamus and basal ganglia.

To examine the level of SNAP-25 mRNA expression in the selected cell populations, we further compared the qRTPCR amplification of SNAP-25 isoform transcripts to β-actin RNA, taken as reference housekeeping gene. Amplification of β-actin RNA transcripts, as determined by Ct values was not significantly different among the different cell types (one way ANOVA, $p = 0.1125$). However, as shown in Fig. 5, panels C and D, the expression level of both SNAP-25a and SNAP-25b transcripts did vary significantly between various brain regions (SNAP-25a, $p < 0.001$; SNAP-25b, $p = 0.0002$, one-way ANOVA). In particular, although the relative levels of the isoform transcripts were similar, the major SNAP-25b RNA transcript, accounting for ~90% of the total SNAP-25 RNA, was significantly lower in GABAergic cerebellar Purkinje cells than other brain regions (Fig. 5D). Moreover, SNAP-25a transcripts in Purkinje cells were also greatly decreased relative to GABAergic neurons of the reticular nucleus and the caudate, as well as hippocampal interneurons and pyramidal neurons (Fig. 5C). Comparing the two major neuronal populations of the cerebellum, the abundance of SNAP-25b transcripts in Purkinje cells was more than an 8.5-fold lower, relative to β-actin, than in neighboring glutamatergic granule cells, despite virtually identical CT values obtained for β-actin RNA (Purkinje cell, 22.22 ±1.26 versus granule cell, 21.77 ± 1.45). Between the neuronal populations surveyed,

GABAergic neurons of the reticular nucleus of the thalamus exhibited a high level SNAP-25 isoform RNA expression with SNAP-25a being significantly increased compared with glutamatergic populations of cerebellar granule cells ($p < 0.01$) and SNAP-25b transcripts at greater abundance than hippocampal pyramidal neurons ($p < 0.05$), indicating that the absolute expression levels of SNAP-25 isoforms were also not tightly correlated with excitatory or inhibitory synaptic transmission. Taken together, these observations suggest that in mature neurons of the major excitatory glutamaterglutamatergic and inhibitory GABAergic populations studied, the predominance of SNAP-25b-containing SNARE complexes neurons is a general, and possibly fundamental, characteristic, regardless of overall abundance of SNAP-25. Moreover, because the isoforms appear equally expressed by both glutamatergic and GABAergic neurons at P7 (Fig. 4D, and see Bark et al., 1995), these data suggest that during development and maturation of neurocircuitry the regulation that drives the predominant expression of SNAP-25b occurs similarly in major populations of excitatory and inhibitory neurons.

3.4 Discussion

Developmental regulation leads to the predominant expression of SNAP-25b in adult glutamatergic and GABAergic neurons

The expression of SNAP-25 in adult rodent brain varies considerably between different neuronal cell groups (Oyler et al., 1989; reviewed in Matteoli et al., 2008). The cellular requirement responsible for the differential abundance of this basic component of the presynaptic exocytotic machinery, however, is less clear, although it likely results from the varied demands in the synaptic physiology of different neurons and their circuitry. For example, early studies based on qualitative in situ hybridization, suggested that while SNAP-25 was expressed robustly by cerebellar granule cells, SNAP-25 transcripts were undetectable in neighboring Purkinje cells (designated as MuBr8 in Branks and Wilson, 1986). Using a more sensitive, quantitative qRT-PCR assay, we show here that the expression of SNAP-25a and 25b RNA transcripts compared to β-actin in Purkinje cells is indeed considerably lower than granule cells, and ranges from 6 to 10.5-fold less than the level detected in the other GABAergic neurons that were examined. Purkinje cells characteristically exhibit a pattern of tonic low frequency firing, accompanied by periodic high amplitude bursts (Loewenstein et al., 2005). Consequently, if SNAP-25 expression is driven by activitydependent induction, the low abundance of SNAP-25 transcripts in Purkinje cells may reflect their intrinsic, relatively low synaptic activity. Nevertheless, our data only reflects mRNA levels in the soma, and not the abundance or activity of SNAP-25 within the presynaptic terminal, which may be also regulated by post-translational

modifications, such as palmitoylation (Hess et al., 1992; Salaun et al.,2005) or phosphorylation (Kataoka et al., 2000; Kataoka et al., 2006; Shu et al., 2008; Pozzi et al., 2008). For example, in the hippocampus the abundance of SNAP-25 protein in presynaptic mossy fiber terminals of dentate gyrus granule neurons, which are highly active and contain a disproportionately large pool of releasable vesicles, appears much higher than in the terminal fields of neighboring pyramidal neurons (Oyler et al, 1989; Boschert et al., 1996), despite apparent lower levels of mRNA transcripts compared to CA3 pyramidal neurons. This suggests that trafficking, as well as functional modifications of this t-SNARE might play yet an additional critical role in the specialization of mechanisms that govern presynaptic neurotransmitter release.

While our results demonstrating the predominant expression of SNAP-25b isoform transcripts among neuronal populations of the adult mouse brain agree with a general shift in alternative splicing accompanying neuronal maturation (Bark et al., 1995), we were surprised to find no evidence for the differential expression of the two isoforms that has been observed previously between other regions of the CNS (Bark et al., 1995; Boschert et al., 1996; Jacobsson et al., 1996; Jacobsson et al., 1999; Jacobsson et al., 1999). Among the brain regions we sampled, the prevalence of SNAP-25a transcripts was remarkably consistent (averaging 9.8% ± 1.7%, S.D.; see Fig. 5B) for neurons selected for either GABAergic or glutamatergic transmitter phenotype. In fact, cerebellar granule

cells showed the most difference between these neuronal populations with SNAP-25a transcripts accumulating to only 7.0% of the total SNAP-25 RNA. One explanation for the discrepancy between our present results and the previous findings is that we selected neuronal populations primarily involved in fast, point-to-point neurotransmission, thus largely excluding neurons that primarily secrete other neurotransmitters and may depend more heavily on SNAP-25a expression (Bark and Wilson, 1994). For example, among the areas of the brain shown to exhibit preferential expression of SNAP-25a into maturity, the pituitary and hypothalamus are chiefly populated by neurosecretory neurons that are characterized by their release of hormones and other neuropeptides (Jacobsson et al., 1996; Jacobsson et al., 1999). Neurons in these areas maybe more comparable to other neuroendocrine cells, such as adrenal gland and pancreatic beta cells, that persistently express high levels of SNAP-25a in the adult (Jacobsson et al., 1994; Gonelle-Gispert et al., 1999; Grant et al., 1999), suggesting that the preference for one isoform may reflect a mechanism that tailors exocytotic machinery to secretory properties. Consistent with this idea, expression of SNAP-25b leads to the greater recruitment of vesicles to the readily releasable pool in hippocampal neurons compared to SNAP-25a (Delgado-Martinez et al., 2007), and similarly stabilizes a larger pool of vesicles for catecholamine secretion in adrenal chromaffin cells (Sorensen et al., 2003). Interestingly, the expression of the isoforms also appears to be responsive to synaptic activity. Depolarization of dentate gyrus granule cells has been shown to induce expression of SNAP-25b rather than SNAP-25a (Hepp et al., 2001),

whereas activation of neurosecretory magnocellular hypothalamic neurons has been reported to increase SNAP-25a expression exclusively (Jacobsson et al., 1999). Selection of a particular SNAP-25 isoform, therefore, may provide a functional advantage in refining the exocytotic machinery necessary for different modes of vesicular release.

Consistent with previous studies (Bark et al., 1995; Boschert et al., 1996), we find a similar developmental profile of SNAP-25 isoforms in GABAergic (GFP_{pos}) and non-GABAergic (GFP_{neg}, glutamatergic) neurons in the cortex with equal representation of SNAP-25a and -25b transcripts in neonates leading to the predominant expression of SNAP-25b in the adult. Interestingly, SNAP-25b is also the predominant isoform expressed in dentate gyrus granule cells (Boschert et al., 1996) that have been shown to simultaneously release both GABA and glutamate (Gutierrez and Heinemann, 2006). Several recent studies have reported, however, that although SNAP-25 was detected initially in interneurons of the developing hippocampus, the expression waned as these GABAergic neurons mature in culture and appeared to be undetectable at these synapses, as well as synapses of other GABAergic neurons in the adult brain (Verderio et al., 2004; Frassoni et al., 2005; Pozzi et al., 2008; for review, see Matteoli et al., 2008). Although the reason for discrepancy between these observations and our previous results demonstrating co-localization of SNAP-25 immunoreactivity with GABAergic markers in several GABAergic neuronal populations (Tafoya et al.,

2006) remains to be resolved, our present findings indicate that SNAP-25b RNA transcripts are, in fact, robustly expressed by GABAergic neurons isolated from cortex, thalamus, caudate, as well as by hippocampal interneurons at a level comparable to that seen in excitatory, glutaminergic neurons. Taken together with other studies (Tafoya et al., 2006; Bronk et al., 2007; Delgado-Martinez et al., 2007), these results provide additional evidence that SNAP-25b is a key component of the neural SNARE complex responsible for both GABAergic and glutamatergic transmission in mature neurons.

Alterations of calcium dynamics in SNAP-25 deficient neuronal cultures

Consistent with the idea that evoked synaptic activity is required to establish network activity between cultured neurons, we found that spontaneous, synchronized calcium oscillations were absent in dispersed hippocampal cultures prepared from *Snap25*$^{-/-}$ mice. These SNAP-25 deficient mutant neurons were, nevertheless, able to generate calcium transients after depolarization. Interestingly, the amplitude of spontaneous synchronous calcium spikes in cultures from heterozygous null mutants, expressing reduced levels of SNAP-25, did not differ substantially from wild type neurons. Moreover, the magnitude of the calcium response evoked in SNAP-25 null mutant neurons by exposure to high K^+ depolarizing media was also equivalent to the responses measured in control *Snap25*$^{+/-}$ and *Snap25*$^{+/+}$ neurons. Previous studies have shown that SNAP-25 is associated with N and P/Q type voltage gated calcium channels (for

review, see Catterall 1999), and specifically impedes calcium currents through P/Q type channels activated in response to action potentiallike stimuli (Zhong et al., 1999). In our experiments, however, we did not observe a significant enhancement of the relative contribution of MCVII toxin sensitive P/Q type channels to the overall calcium response in *Snap25*$^{-/-}$ compared to control neurons. This suggests that the modulation of these calcium channels by SNARE protein interactions does not occur in the soma, but may be limited to presynaptic terminals, which lie beyond the level of resolution achieved in these experiments. In contrast to these results, Matteoli and colleagues have reported a *Snap25* genotype-dependent difference in calcium responsivity with higher peak calcium responses evoked from hippocampal neurons prepared from homozygous *Snap25* null mutants compared to wild type, and intermediate values from neurons heterozygous for the null mutation (Pozzi et al., 2008). One possibility that could contribute to these different findings may be the variability seen in the viability of SNAP-25 deficient neurons in culture (see Washbourne et al., 2002; Bronk et al., 2007; Delgado-Martinez et al., 2007). In an effort to control for this variability, we averaged the mean calcium peak response exhibited by 6–8 individual cultures (after assaying 5–10 neurons per field) for each genotype. Moreover, to control for differences in the complexity of neurite extension that is evident between cultures, and more importantly genotypes, we restricted our measurements to fura-2a responses imaged over cell bodies, thus avoiding the contribution of calcium transients in dendrites.

Astrocytes have been proposed to join with presynaptic terminals and postsynaptic spines to form a "tripartite synapse," that enables bidirectional communication between glia and neurons (for review, see Verkhratsky 2006). Indeed, in most cultures of SNAP-25 expressing neurons, neuronal depolarization was accompanied by clear increases in astrocyte Ca^{2+} oscillatory activity. This correlation could be due to a number of factors, including direct effects of K^{+} triggering depolarization on astrocytes. Nevertheless, since astrocytes do not express SNAP-25, but utilize the independent t-SNARE homologue SNAP-23 (Parpura et al., 1995), this deficit in astrocyte Ca^{2+} responsiveness in SNAP-25 deficient cultures provides further evidence for the role of AP-dependent synaptic transmission in neuronal-glia communication.

3.5 Conclusions

Overall, our results are consistent with the idea that SNAP-25b serves as the predominant t-SNARE responsible for action potential-dependent neurotransmission in the major excitatory glutamatergic and inhibitory GABAergic neurons in the mature brain. In addition, we conclude that while deficits in SNAP-25 do not selectively dysregulate specific voltage-gated calcium channels at the soma, this neural SNARE component is needed to maintain normal synaptic activity that is reflected by calcium signaling between neurons and within a neural-glial network.

3.6 Methods

Animal procedures

Heterozygote *Snap25* null mutant mice (JAX strain designation B6.129X1-*Snap25*tm1Mcw/J; see Washbourne et al., 2002) have been maintained by brother:sister mating after 7 backcross generations to C57Bl/6 at the UNM HSC Animal Resource Facility. To prepare neuronal cell cultures, *Snap25* homozygote null mutants (*Snap25*$^{-/-}$), heterozygote *Snap25*$^{+/-}$ and wild type (*Snap25*$^{+/+}$) fetuses were collected from timed pregnant dams of heterozygote matings. At E17-E18 (plug date, day 0) pregnant animals were killed by rapid cervical dislocation and decapitation as described previously (Washbourne et al., 2002; Tafoya et al., 2006). Fetuses were removed sequentially from the uterus, and *Snap25*$^{-/-}$ fetuses were initially identified by the absence of a response to a pinch to the hindlimb. PCR genotyping (Washbourne et al., 2002) was used to confirm null *Snap25*$^{-/-}$ mutants, and to distinguish between heterozygote *Snap25*$^{+/-}$ and homozygote *Snap25*$^{+/+}$ fetuses that served as control littermates. Pups were quickly decapitated and their brains were removed and placed in ice-cold PBS. For FACS analysis and laser capture microscopy studies (see below for Methods), mice were euthanized with phenobarbital. All procedures were performed in accordance with guidelines of the University of New Mexico Health Sciences Center Laboratory Animal Care and Use Committee, and the National Institutes of Health.

Imaging of intracellular Ca^{2+} transients

Hippocampal neurons were isolated from individual E17.5 fetal mice and grown as dispersed mixed cell cultures plated on poly-L-lysine/laminin-coated 12 mm coverslips (four coverslips per animal; 50,000 cells/coverslip) for 9–11 days (9–11 DIV) as described previously (Washbourne et al., 2002). Cytosolic Ca^{2+} levels were assessed using the high-affinity ratiometric indicator Fura-2. Cultures were loaded at room temperature with 3 μM Fura-AM for 20 min in HEPES buffer (130 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 11 mM Glucose, 10 mM HEPES pH 7.6) and then rinsed for 20 min in HEPES to allow for deesterification of indicator. Cultures were then transferred to the recording chamber and superfused with HEPES at 2 ml/min at room temperature. Cultured were allowed to equilibrate to the recording conditions for 20 min before recording was begun. Depolarization-induced Ca^{2+} increases were evoked by rapid complete exchange of the chamber contents with 55 mM K^+ solution. 10 min intervals in normal HEPES buffer were maintained between repetitive challenges. In the absence of any inhibitors, this procedure produced reproducible Ca^{2+} responses throughout the time course of these experiments. Antagonists were applied to cultures 5 min before the onset of K^+ challenges, and maintained in the recording solutions thereafter. Fura-2 excitation was achieved using 350/380 nm pairs (40 ms each) delivered from a monochromator (TiLL Photonics GmbH, Grafeling, Germany) via a 40 × WI objective (Olympus, N.A. 0.8). Fluorescence emission (510 nm) was detected using an interline transfer cooled CCD (TiLL Imago). Image pairs were background-subtracted and then ratioed (Till Vision v 4.0).

Separation of GAD67-GFP (ΔNeo) cells by Fluorescence Activated Cell Sorting

Cerebral cortices were washed twice in PBS and then incubated in a solution containing papain (2 mg/ml; Sigma, St. Louis, MO) and Hibernate A media (without CaCl2; BrainBits LLC, Springfield, IL) for 30 minutes at 30°C. Digested tissue was then transferred to Hibernate A alone followed by mild trituration through both wide-bore and fine-tipped pipettes. Prior to flow cytometry, the cells were filtered and resuspended in 5 ml of ice-cold PBS.

Flow cytometry was performed using the MoFlo High-Performance Cell Sorter (Dako Inc., Fort Collins, CO) equipped with a 488 nm excitation laser and a 530–540 nm band pass filter. eGFP expressing cells were sorted at a rate of 1000 events/sec through a 100 µm nozzle. Gating threshold parameters were selected was based on optimal measurements of side scatter (SSC) and GFP fluorescence. Two separate fractions, either GFP-positive or GFP-negative cells, were collected for each cortical sample.

The isolated cells were centrifuged at 500 × g for 5 min, supernatant was removed, and tissue pellets were homogenized in 4 M guanidinium thiocyanate (GTC), 50 mM Tris pH 8.5, 10 mM EDTA, 0.5% sarcosyl, and 200 mM β-mercaptoethanol. After mixing in 1/10 volume of 2 M sodium acetate (pH 4.0), RNA was extracted using 1/5 volume of a 24:1 chloroform/isoamyl alcohol

mixture and 1.0 volume of acid phenol (pH 4.3). Samples remained on ice for 20 min, followed by centrifugation at 4°C for 15 minutes. The aqueous phase was collected and the RNA was precipitated by addition of isopropanol (1.0 volume). Samples were placed at -20°C for 1 hour, centrifuged, and washed with 70% ethanol before resuspension in DEPC-treated water. RNA samples were then stored at -80°C until use.

Laser capture microdissection

While irreversible cross-linking fixatives, such as paraformaldehyde, provide excellent conservation of GFP fluorescence, throughout subsequent tissue processing, it greatly compromises RNA integrity. Therefore, as an alternative, we used the reversible cross-linking fixative, DSP to balance preservation of mRNA levels while retaining detectable GFP fluorescence in the tissue (Xiang et al., 2004). After euthanization with phenobarbital, mice were transcardially perfused with 0.1 M phosphate buffer (PB) to flush out brain vasculature, followed by a 1 mg/ml solution of dithiobis(succinimidyl)propionate (DSP; Pierce Biotechnology, Rockford IL) in 0.1 M PB. To avoid precipitation of DSP, a 10× stock solution made in DMSO was added slowly to 0.1 M PB, and filtered just before use. After perfusion, the brains were removed and placed in DSP/0.1 M PB solution overnight at 4°C for postfixation. Brains were cryoprotected by immersion in 30% sucrose for 18–24 hours at 4°C, and embedded in Tissue Tek OCT compound (Sakura Finetek, Torrance, CA). 10 μm coronal sections were

cut using a Microm HM 550 cryostat set at -30°C (Richard-Allan Scientific, Kalamazoo, MI), mounted on uncoated glass slides, and stored at -80°C until use.

For laser capture microdissection, neurons from transgenic GAD67-GFP (ΔNeo) mice were harvested using a Pixcell II apparatus (Molecular Devices, Sunnyvale, CA) connected to a Nikon microscope using a 40× objective (N. A., 0.6) and a FITC filter set. Before microdissection, the sections were dehydrated by immersion through 70%, 95%, and 100% ethanol (30 sec each), followed by xylene (5 min), and final air-drying (10 min). GABAergic cells from different brain regions were identified by epifluorescence, and pyramidal neurons of the hippocampal CA1 region and granule cells from the cerebellum were identified by their distinctive cellular morphology using phase contrast optics. Pools of approximately 50 individually dissected cells from each anatomical region were captured on a single CapSure® HS LCM Cap (Molecular Devices) using multiple pulses at a laser power setting of 90 mW, a spot size of 7.5 μm, and duration of 0.1 msec. Each pool of cells, collected from a single animal, was considered as a single, individual sample. To isolate total RNA extracts free from genomic contamination, we used reagents and protocols of the PicoPure RNA isolation kit (Molecular Devices) and the RNase-free DNase kit (Qiagen, Valencia, CA).

Complementary DNA synthesis and quantitative PCR assay

Complimentary DNA (cDNA) was synthesized using 25 pmol oligo(dT)12–18 as a primer (USB, Cleveland, OH) and Moloney murine leukemia virus (MMLV-I; USB) reverse transcriptase using reagents provided by the manufacturer. Briefly, the entire RNA sample was incubated with the primer at 75°C for 5 min, cooled on ice and added to a reaction buffer containing 100 units of reverse transcriptase, M-MLV Reaction Buffer (diluted to 1×, supplied by USB; final concentration containing 50 mM Tris-HCl, pH 8.3, 79 mM KCl, 3 mM MgCl2, 10 mM DTT), and 0.5 mM dNTPs in a volume of 25 µl. The samples at 42°C for 30 min, followed by heat inactivation of the reverse transcriptase at 95°C for 5 min.

Quantitative real-time PCR (qRT-PCR) was carried out on cDNA samples using SYBR green master mix (SuperArray, Frederick, MD). The primer set for VGAT was obtained from SuperArray. Primer sets for VGLUT1, β-actin, and those specific for SNAP-25 isoform transcripts (using a pan SNAP-25 forward with either SNAP-25a [Grant et al., 1999] or SNAP-25b reverse primers), shown in Table 2, were designed or evaluated using software and services from Integrated DNA Technologies (Coralville, IA). Real time quantitative PCR was carried out in a ABI 7000 Sequence Detection System real-time PCR thermocycler (Applied Biosystems, Foster City, CA, USA), under the following cycling parameters: 50°C for 2 min, followed by 95°C for 10 min, then 45 cycles (95°C for 15 sec; 64°C for

45 sec) preceding a dissociation curve. All reactions were performed in triplicate, and each experiment was independently repeated a minimum of three times.

The SNAP-25 isoform primer sets were first evaluated by performing quantitative real-time PCR to generate a standard curve on plasmid DNAs containing cDNAs for the entire open-reading frame of each isoform mRNA (Fig. 6A), demonstrating that the isoform primers were both specific and amplified SNAP-25 isoform sequences at equivalent rates. Similarly, the isoform-specific primers were tested and found to specifically amplify cDNA prepared from COS 7 cells transfected with plasmids encoding either SNAP-25a or 25b transcripts (Fig. 6B). To assess the ability to quantify SNAP-25 isoforms from brain tissue, we also compared amplification from cDNAs prepared from cortex of *Snap25*$^{+/+}$ and a *Snap25* knockin mutant (*Snap25tkneo/tkneo*) that overexpresses SNAP-25a transcripts (Bark et al., 2004). As shown in Fig. 6C, qRT-RCR was readily able to distinguish a 7.5-fold and 5-fold greater expression of SNAP-25a transcripts in young P24 (post-weaning) and adult (P124) knockin mice, respectively, relative to wild type, which is comparable to level of overexpression of SNAP-25a transcripts previously determined for these mice based on isoform-specific cleavage by restriction endonucleases of total SNAP-25 PCR amplified cDNA (see Bark et al., 2004). Taken together, these results demonstrated that the RT-PCR assay with these primers provided a quantitative measure of the relative expression of transcripts encoding the two SNAP-25 isoforms.

Relative transcript levels were calculated from data within the linear range of cDNA amplification, as determined automatically by the instrument software. Within each sample, a $2^{\Delta Ct}$ or $2^{\Delta\Delta Ct}$ analysis method was used to compare the expression levels of target genes after normalizing to amplification of β-actin transcripts as a housekeeping gene. In the series of experiments evaluating SNAP-25 isoform expression in cells harvested from different brain regions by LCM (Fig. 5), the Ct values for β-actin were not significant between cell types (n = 6 samples of each cell type, one-way ANOVA, $p = 0.1125$), indicating that the β-actin levels could be taken as a reference to normalize SNAP-25 transcript levels between these brain regions. For each sample, the absence of amplification from genomic DNA was confirmed by omitting reverse transcriptase during cDNA synthesis before qRT-PCR. Background signal in negative control samples was defined as not detectable based either by failing to cross the detection threshold automatically set by software parameters, or if the amplification was >10 cycles beyond the Ct value of signal found in experimental or positive control samples. Data was analyzed using Prism 4.03 (GraphPad Software) as group means with a Student's t-test or one-way ANOVA.

3.7 Figure Legends

Figure 3.1. Ca^{2+} dynamics in hippocampal cell cultures.

Panel A, Spontaneous synchronized cytoplasmic Ca^{2+} oscillations in *Snap25*$^{+/+}$ hippocampal neurons were abolished by addition of TTX (1 μM). Each trace is from a different neuron within the same culture dish. The inset shows a segment of the recording (prior to TTX, denoted by asterisk) at an expanded time base to show more clearly the synchrony of events. Panel B, Recordings from a culture derived from a homozygote *Snap25*$^{-/-}$ fetus under identical conditions as illustrated in A. Spontaneous Ca^{2+} transients in *Snap25*$^{-/-}$ neurons were very rare and a single event in one neuron is shown at an expanded time base in the inset. Panel C, Depolarization with 55 mM K^+ (arrow) led to a sustained Ca^{2+} elevation in neurons from both. *Snap25*$^{+/+}$ (left panel) and *Snap25*$^{-/-}$ (right panel) cultures. TTX (1 μM) was included in both preparations, prior to K^+ application. Panel D, Astrocyte Ca^{2+} oscillations from the same culture dishes illustrated in Panel C. Under control conditions, spontaneous events were observed in both *Snap25*$^{+/+}$ (left panel) and *Snap25*$^{-/-}$ (right panel) cultures. Following K^+ application, an increase in frequency and amplitude of astrocyte events was observed in astrocytes from *Snap25*$^{+/+}$ but not in the *Snap25*$^{-/-}$ preparation. A dashed line is drawn near the initial peak of neuronal Ca^{2+} increases in the *Snap25*$^{+/+}$ preparation, to emphasize the relationship between neuronal and astrocyte signals. A similar relationship was not apparent in the *Snap25*$^{-/-}$ culture. (See Fig. 3 for group astrocyte data).

Figure 3.2 Summary of neuronal Ca^{2+} responses.

Panel A depicts summary data from 7 coverslips for each genotype, showing the mean amplitude of spontaneous Ca^{2+} oscillations were not different between *Snap25*$^{+/+}$ (+/+) and *Snap25*$^{+/-}$ (+/-) cultures. Panel B, amplitude of Ca^{2+} elevations evoked by K^+-depolarization in *Snap25*$^{+/+}$ (+/+), *Snap25*$^{+/-}$ (+/-) and *Snap25*$^{-/-}$ (-/-) cultures (n = 7,6,8, respectively). TTX (1 μM) was present throughout K^+ challenges. Panel C shows effects of sequential bath application of Ca^{2+} channel blockers in 55 mM KCl containing media to assess the role of voltage-gated calcium channels in depolarization triggered Ca^{2+} elevations of pooled results from *Snap25*$^{+/-}$ and *Snap25*$^{+/+}$ (+/- and +/+) compared to *Snap25*$^{-/-}$ (-/-) neurons. Responses were normalized to 55 mM K^+ challenges in control buffer. Con, control (TTX only), Nim, nimodipine (10 μM), conotoxins GVIA and MVIIC (1 μM).

Figure 3.3 Summary of astrocyte Ca^{2+} oscillation responses.

Panel A, Frequency of Ca^{2+} oscillations in astrocytes under control conditions (open bars) and during a 2 min time window following application of 55 mM K^+ (filled bars). Significant increases were observed in *Snap25* wild type and heterozygous control cultures, in contrast to a decrease in the *Snap25*$^{-/-}$ cultures. Panel B, The mean amplitude of individual astrocyte Ca^{2+} oscillations was significantly increased following K^+ application in control cultures. Each bar represents mean ± SEM from 5 different cultures. Only astrocytes that displayed spontaneous events prior to K^+ were included in the analysis and responses from 3–5 cells per dish were averaged before calculating group means. (** $P < 0.005$; * $P < 0.05$).

Figure 3.4 Developmental regulation of SNAP-25 isoform expression in GABAergic neurons.

Panels A and B show a pair of graphs comparing cell populations prepared from acutely dissociated adult cortex of GAD67-GFP (ΔNeo) and nontransgenic wild type mice, respectively, after fluorescence-activated cell sorting (FACS). The selectivity of the fractionation is demonstrated by the distinct population of GFP-positive cells (dashed oval, panel A) that was readily distinguished in cells derived from GAD67-GFP (ΔNeo) mice, but was absent in preparations from wild type animals (panel B). The arrows in A indicate the two sorted cell populations, GFP-positive (green arrow) and -negative (black arrow), used for molecular analysis. Panel C shows the relative levels of SNAP-25a (solid bar) and SNAP-25b (white bar) RNA transcripts in adult neurons from GFP-positive (GFP_{pos}, n = 4) and -negative (GFP_{neg}, n = 2) populations. Panel D, SNAP-25 isoform RNA transcript expression in sorted immature neurons obtained from cortex of P7 neonates (GFP_{pos}, n = 7; GFP_{neg}, n = 4). Note that in contrast to the earlier developmental time point where comparable levels of the two isoform transcripts are expressed in both GFP_{pos} and GFP_{neg} cell populations, mature neurons of both populations express predominantly the SNAP-25b transcript.

Figure 3.5 SNAP-25 isoform expression varies between anatomical regions of the adult brain.

GFP-expressing cells from coronal sections of adult (P50) transgenic *GAD67-GFP (ΔNeo)* mouse brains were identified by epifluorescence and isolated by laser capture microdissection (LCM) from the reticular nucleus of the thalamus (Ret. Nuc.), caudate, cerebellum (Purkinje cells), and CA1 region of the hippocampus (Hp interneuron). In addition non-GFP expressing glutamatergic neurons collected from the pyramidal layer of the hippocampus (Hp Pyramidal) and granule layer of cerebellum (Cb Gran. Cell). The total RNA extracted from microdissected samples was assayed by real-time qRT-PCR analysis to assess transcript levels relative to β-actin as a control gene, as described in the Methods. Panel A is a representative micrograph of a GFP-expressing cell (arrowhead and hashed outline) before and after LCM, visualized with both phase contrast (left) and a FITC filter set (right). Note how neighboring cells (arrows) and tissue remain intact during the procedure. B, Histogram shows the relative ratio of SNAP-25a (solid bar) and 25b (white bar) RNA expression in selected brain regions (n = 6 pools taken from individual brains). Note that although, in all cases, SNAP-25b expression is significantly higher than SNAP-25a (***$P < 0.001$, Student's t-test), the ratio of isoform expression between anatomical regions does not change (n. s.; One-way ANOVA with Tukey post hoc comparison). Throughout the areas surveyed, however, the overall mRNA expression of SNAP-25a and SNAP-25b did vary widely (shown in Panels C and D, respectively). Statistical analysis with a one-way ANOVA and Tukey post hoc

comparisons of total SNAP-25a and SNAP-25b transcript expression was carried out for each anatomical region (n = 3, except for cerebellar granule cells, n = 6). Asterisks denote significance of the difference between the level of SNAP-25 transcripts of each region compared to cerebellar Purkinje cells (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$); additional significant differences in isoform transcripts between GABAergic neurons of the reticular nucleus (Ret. Nuc.) and non-GABAergic neurons of the hippocampus (Hp Pyramidal) and cerebellum (Cb Gran. Cell) are also indicated.

Figure 3.6 Quantitative real-time PCR assay of SNAP-25 isoform transcripts.

Quantitative RT-PCR demonstrated specific and equivalent amplification of SNAP-25 isoform sequences using primers designed to exploit nucleotide sequence differences between SNAP-25a and 25b (see Table 2). Panel A, Calibration curve of quantitative RT-PCR performed using primers sets on SNAP-25 isoform cDNA. Ct values (triplicates ± SEM) obtained for each primer/cDNA plasmid pair were plotted versus the amount of DNA template on a log scale to demonstrate the linear relationship between amplification and DNA input. Robust and equal amplification of each SNAP-25 isoform cDNA was detected only with the appropriate primer set and corresponding plasmid (SNAP-25a: 25a/25a, blue closed squares; and SNAP-25b 25b/25b, orange open squares). Only non-specific, negligible amplification (CT values >30) was obtained from non-corresponding primer/plasmid sets (e.g. 25a/25b, green triangles; 25b/25a red circles). Panel B, RT-PCR performed on RNA of transfected cells. COS7 cells (1.5 × 105 cells/well of a 12 well plate) were transfected with equivalent molar amounts (~1 µg/well) pCDNA3 expression plasmids bearing SNAP-25 isoforms (Bark et al., 1995), or the empty vector (pCDNA), using Lipofectamine (Invitrogen, Carlsbad CA, USA). cDNA prepared from RNA (1 µg) extracted from the transfected or untransfected (Untrans) control cells was amplified using the indicated isoform specific reverse primer, either individually or together (25a+25b), by conventional end-point reverse transcriptase PCR (40 cycles, see Methods). Amplification using a primer set to S12 rRNA protein transcripts served as a positive control. The size of the isoform

specific amplicons (SNAP-25a 149 bp; SNAP-25b, 176 bp) is due to the different positions of isoform-specific reverse primers relative to the common forward primer, pan*SNAP25*. The lack of a band in the mismatched primer/template lanes reflects the specificity of the PCR reaction. Panel C, Quantitative RT-PCR using total RNA preparations from cortex of wild type (WT, white bar) and SNAP-25a overexpressing knockin mutant (Snap25tkneo, black bar) mice. The relative overexpression of SNAP-25a transcripts in the homozygous mutant (Tkneo/Tkneo) samples, expressed as ΔΔCt (25a/25b) normalized to wild type (WT), was calculated from the SNAP-25 isoform amplification relative to β-actin (ΔCt value) and then deriving the relative ratio of their amplification rates (ΔΔCt). The ratio of SNAP-25a to SNAP-25b (e.g. 25a/25b) obtained from the mutant was then normalized to wild type (set at 1.0) to obtain a fold increase of SNAP-25a expression in these mutant mice. This increased level of SNAP-25a isoform transcripts in the mutant cortex RNAs, seen in both young (P24) and adult (P124), is consistent with values previously reported for these mice (Bark et al., 2004) confirming the specificity of each primer set and their use in assaying the expression of SNAP-25 isoforms in brain tissue.

3.8 Figures

Figure 3.1

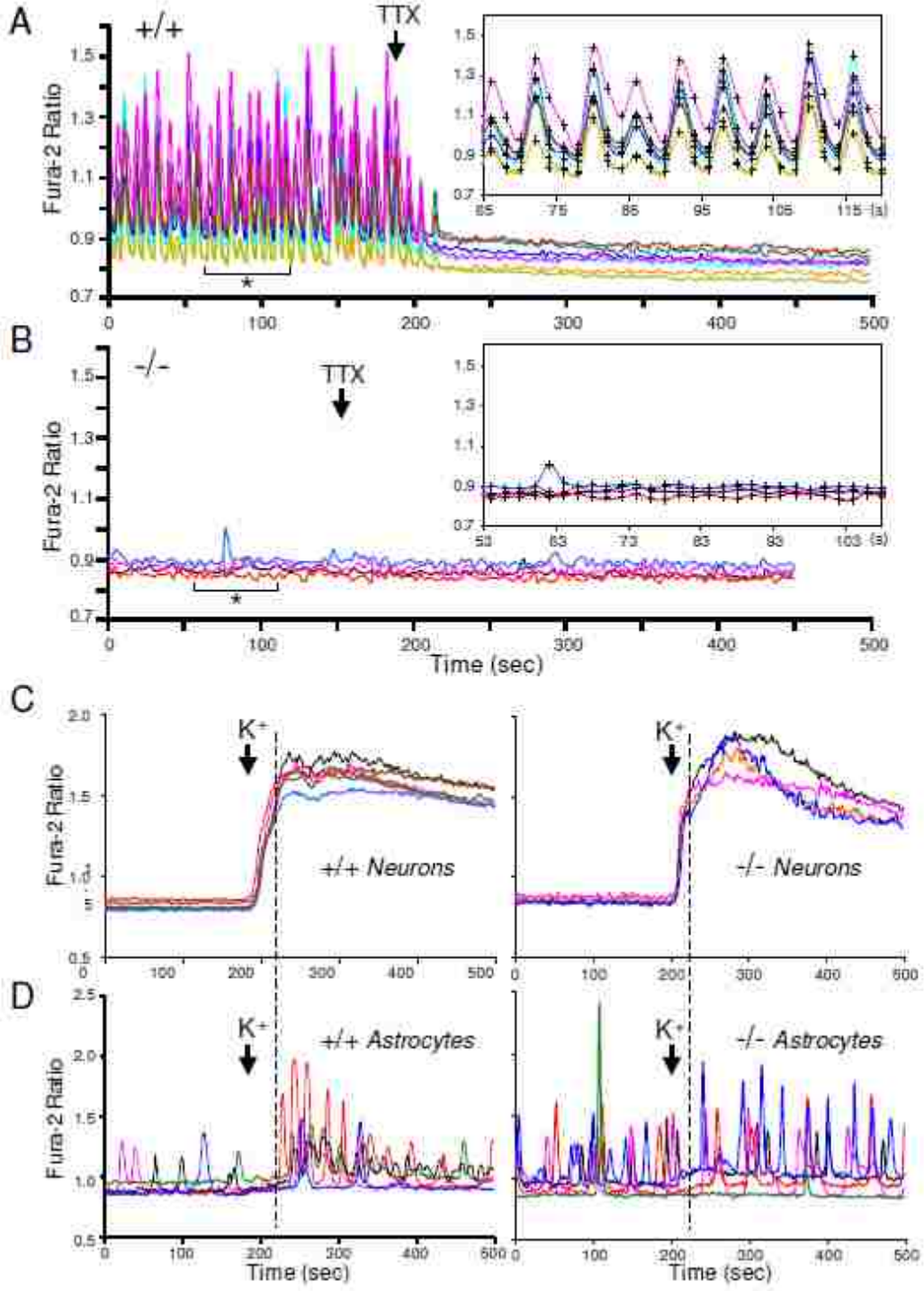
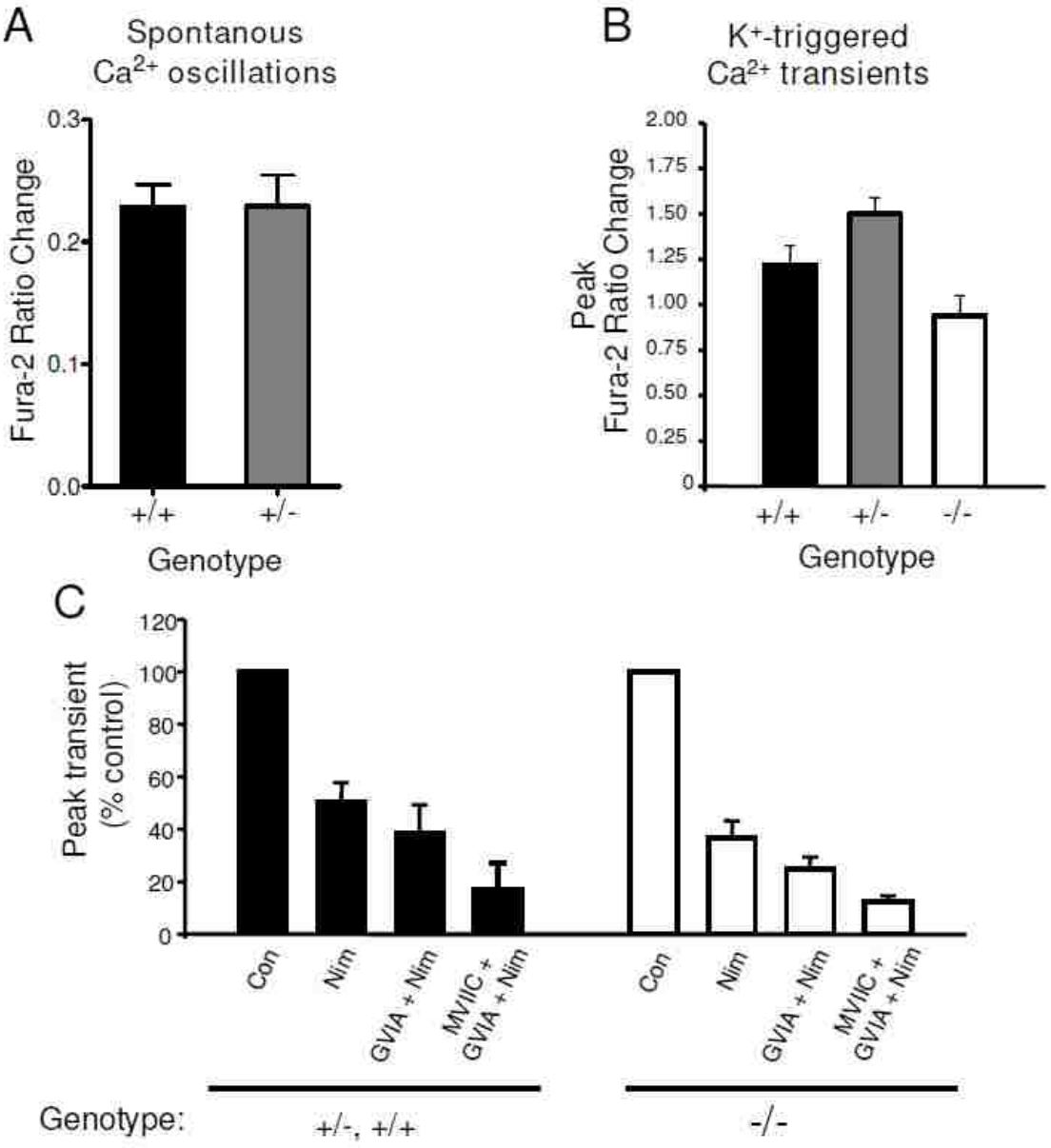


Figure 3.2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Spontanous
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Fura-2 Ratio Change
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0.2
0.1
0.0
+/+
+/-
Genotype
B
K+-triggered
Ca2+ transients
Peak
Fura-2 Ratio Change
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0
+/+
+/-
-/-
Genotype
C
Peak transient
(% control)
120
100
80
60
40
20
0
Con
Nim
GVIA + Nim
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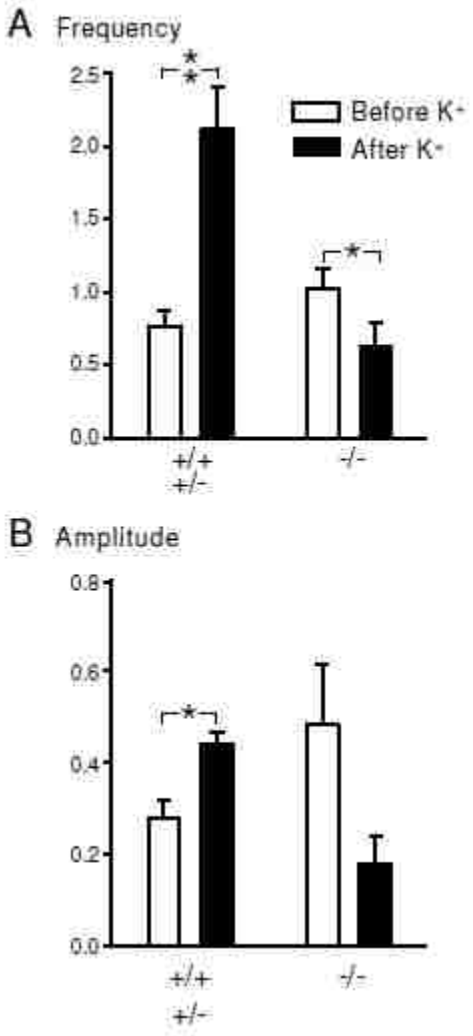
Genotype:
+/-, +/+
-/-

Figure 3.3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2.0
1.5
1.0
0.5
0.0
Before K+
After K+
*
*
*
+/+
+/-
-/-
B Amplitude
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0.6
0.4
0.2
0.0
*
+/+
+/-
-/-

Figure 3.4

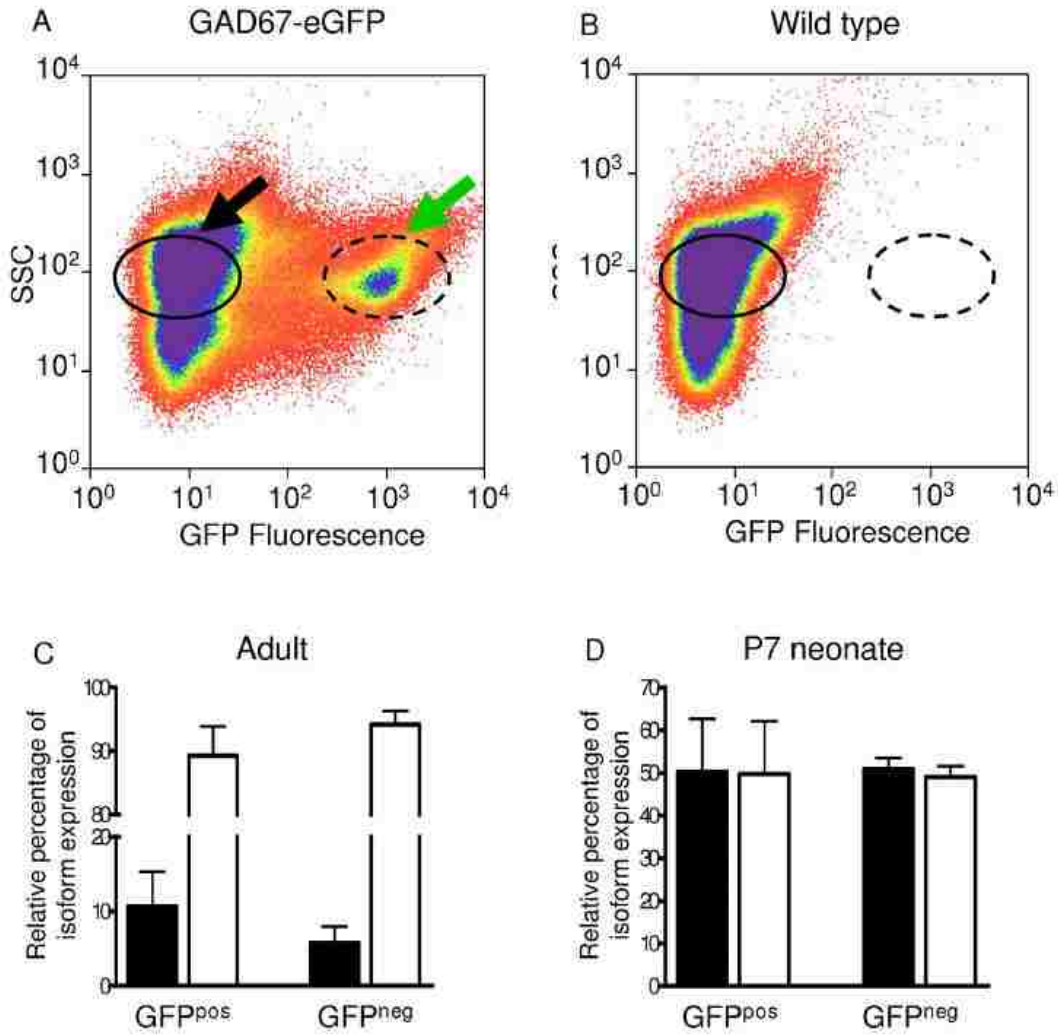
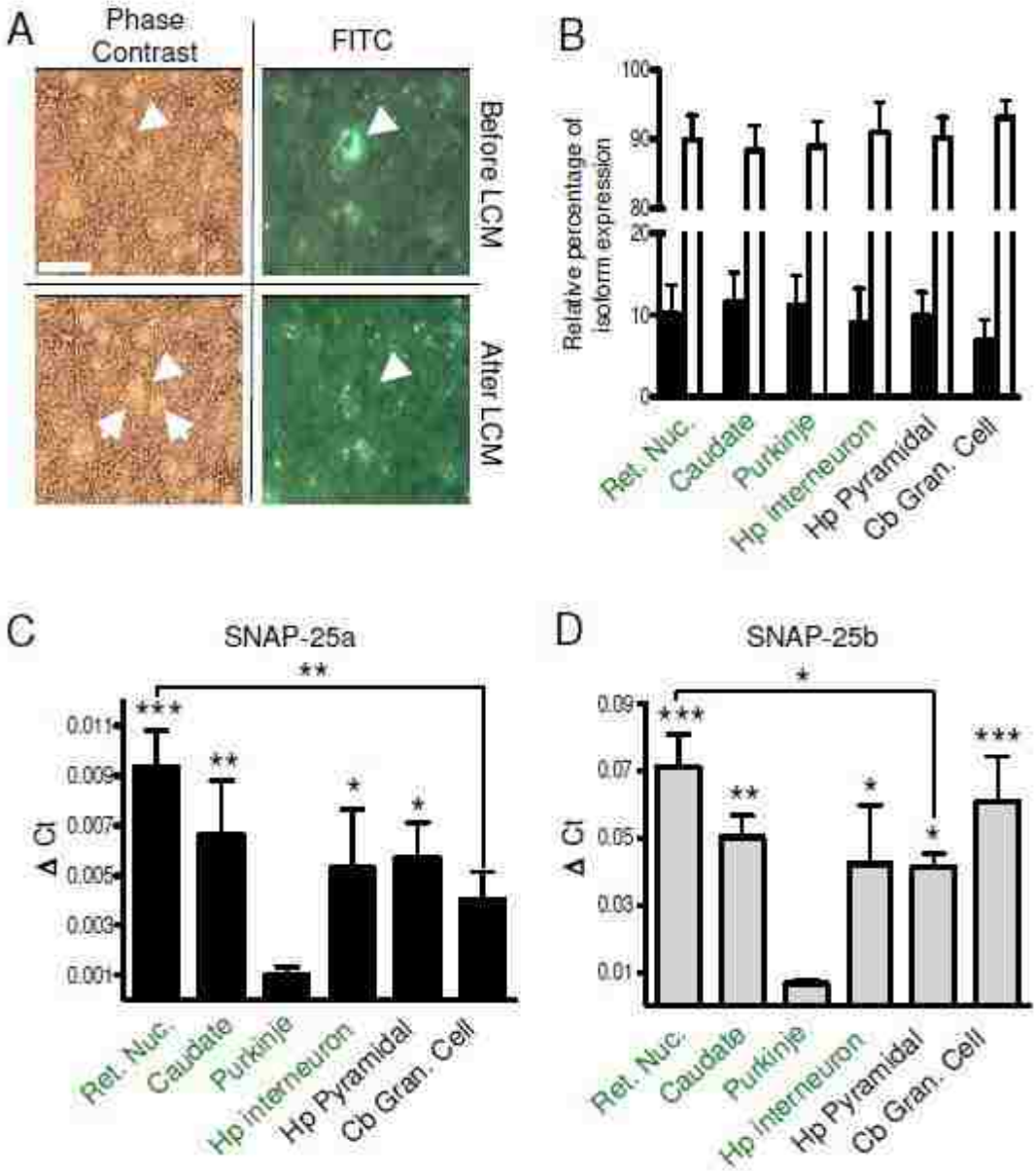

A
GAD67-eGFP
B
Wild type
SSC
GFP Fluorescence
GFP Fluorescence
C
Adult
D
P7 neonate
Relative percentage of isoform expression
Relative percentage of isoform expression
GFPpos
GFPneg
GFPpos
GFPneg

Figure 3.5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Before LCM
After LCM
B
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80
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10
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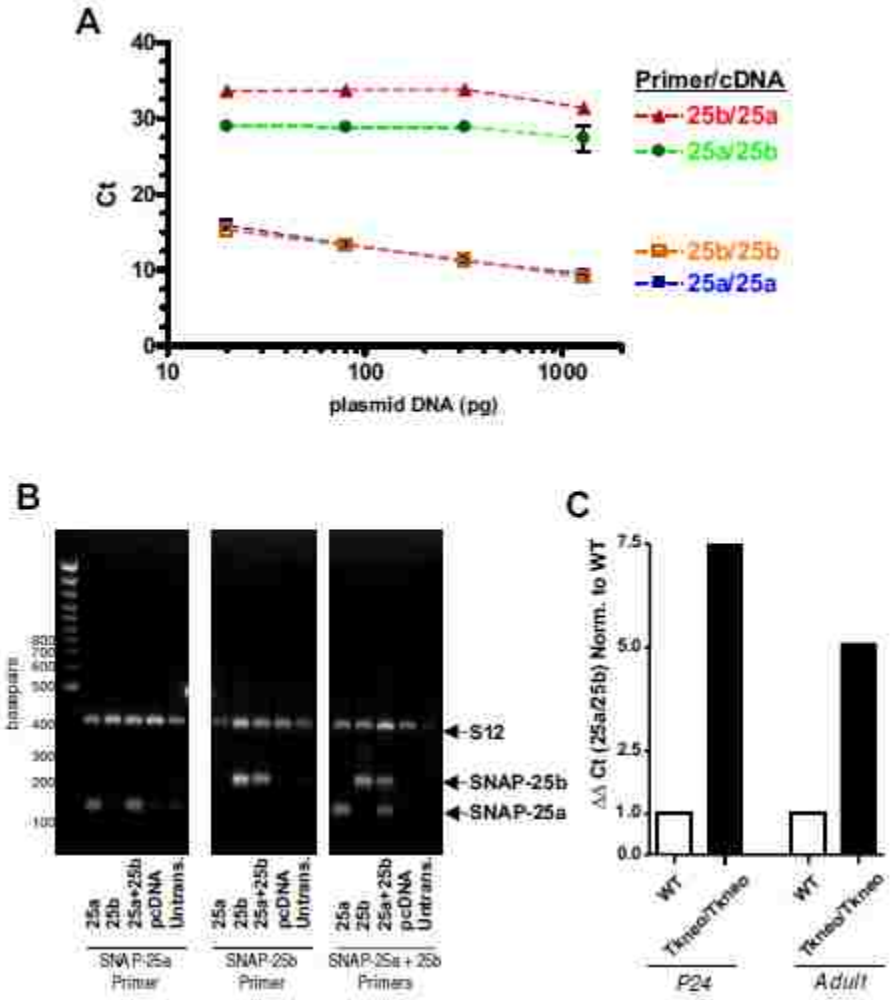
Caudate
Purkinje
Hp interneuron
Hp Pyramidal
Cb Gran. Cell
C
SNAP-25a
Δ Ct
0.011
0.009
0.007
0.005
0.003
0.001
D
SNAP-25b
0.09
0.07
0.05
0.03
0.01

Figure 3.6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Ct
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Primer/cDNA
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25a/25b
25b/25b
25a/25a
B
basepairs
S12
SNAP-25b
SNAP-25a
25a
25b
25a+25b
pcDNA
Untrans.
SNAP-25a Primer
SNAP-25b Primer
SNAP-25a + 25b Primers
C
ΔΔ Ct (25a/25b) Norm. to WT
WT
TkneoTkneo
P24
Adult

3.9 Tables

Table 3.1 - Relative percentage of VGLUT1 and VGAT mRNA levels compared to β-actin expression.

Fluorescence-activated cell sorting			
Sample	*GFP Phenotype*	*VGLUT1*	*VGAT*
Immature cortical cells	Negative	2.37%	n. d.*
	Positive	n. d.	7.89%
Adult cortical cells	Negative	3.91%	n. d.
	Positive	n. d.	9.51%
Laser capture microdissection			
Region	*GFP Phenotype*	*VGLUT1*	*VGAT*
Reticular Nucleus	Positive	n. d.	12.68%
Caudate	Positive	n. d.	14.07%
Purkinje cells	Positive	n. d.	15.39%
Hippocampal interneurons (CA1)	Positive	n. d.	12.45%
Hippocampal pyramidal cells (CA1)	Negative	11.95%	n. d.
Cerebellar granule cells	Negative	13.56%	n. d.

* n. d. (not detected) is considered less than 0.001% (i.e. Ct value > 10 cycles) of gene expression in comparable amounts of whole brain samples (see Materials and Methods).

Table 3.2 - Sequence of oligonucleotides

Primer	*Bases*	*Sequence (5'-3')*	*Reference*
VGLUT1 forward	747–776	AGGAGGAGCGCAAATACATTGAGGATGCCA	BC054462
VGLUT1 reverse	866-837	TGATGGCATAGACGGGCATGGACGTAAAGA	
β-actin forward	1033–1062	TGCTCTGGCTCCTAGCACCATGAAGATCAA	NM007393
β-actin reverse	1231-1202	AAACGCAGCTCAGTAACAGTCCGCCTAGAA	
Pan SNAP-25 forward	58–82	CAGCTGGCTGATGAGTCCCTGGAAA	AB003991
SNAP-25a reverse	207-172	TTGGTTGATATGGTTCATGCCTTCTTCGACACGATC	AB003992
SNAP-25b reverse	267-234	CACACAAAGCCCGCAGAATTTTCCTAGGTCCGTC	

3.10 Authors' Contributions

LCRT prepared neuronal cultures, designed and carried out the molecular analysis of GABAergic and glutamatergic neurons, and drafted the manuscript. CWS performed the calcium imaging experiments. YY and KO provided the GAD67-eGFP mice. MCW was responsible for the overall design of the study, participated in the calcium imaging and helped to draft the manuscript. All authors read and approved the final manuscript.

3.11 Acknowledgements

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4. Discussion

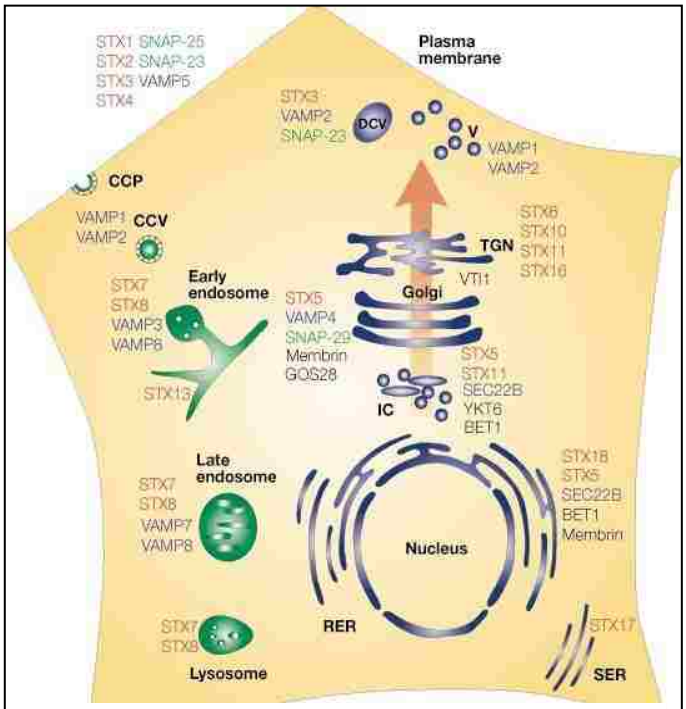
The various stages of vesicle budding, trafficking, and fusion have been widely studied and detailed in many different organisms, resulting in the identification of an array of conserved and divergent proteins involved in these processes. Defining subsets or functional groups of proteins and determining their specific functional roles in regulated release from different cell types, however, remains elusive. A deeper understanding of the mechanisms behind various forms of neurotransmission relies on the ability to identify the various obligate and modulatory components used to carrying out regulated secretion at distinct synapses. The heterotrimeric SNARE complex, containing SNAP-25, syntaxin 1a, and VAMP-2, is required for the regulated release of several diverse neurotransmitters and neuropeptides, suggesting a specialized role that is central to the diverse chemical signaling and communication occurring throughout all neural and neuroendocrine pathways of the body. However, despite powerful evidence confirming the obligate nature of this fundamental core complex in the calcium-triggered secretion of different cell types, it was uncertain whether this requirement was maintained in GABAergic neurons.

In the current study, we have shown evidence that supports the model that a conserved SNARE complex underlies the mechanism of evoked neurotransmitter release. We proposed the hypothesis that SNAP-25 is a requisite component of the core fusogenic complex essential for evoked GABAergic neuroexocytosis

and the differential isoform expression of this t-SNARE provides the molecular underpinnings for the distinctive properties of inhibitory transmission. Our hypothesis was tested by experimental methods that provided evidence which established SNAP-25 expression in GABAergic terminals. Next, we examined the requirements of this t-SNARE for AP-dependent recycling of GABAergic vesicles. Finally, we characterized the relative ratio of SNAP-25 isoform expression in GABAergic neurons. Overall, we provided compelling evidence, in collaboration with results from other labs, which extends this requirement to major inhibitory neurons of the brain by revealing the dependence of evoked GABAergic transmission on SNAP-25 expression. The results of our research and additional interpretations of these findings are presented below.

The experimental design, results, and thorough discussion of these findings have been included within the preceding chapters. Therefore, in this final chapter, the results will only be briefly recapitulated and a more extensive

Figure 4.1 Various SNARE complexes expressed in eukaryotes



Schematic drawing demonstrating the various SNARE complexes specialized to carry out vesicle targeting and fusion within specific trafficking pathways. Image is reproduced from Chen and Scheller 2001.

discussion of the global implications that these results have and how they support or challenge the unifying hypothesis that was originally developed. In addition, several arguments will be provided that relate our findings to established results and to advance potential mechanisms behind these observations. Finally, potential limitations of the experimental design utilized will be discussed as well as prospective future directions in both research design and clinical application that may be undertaken from this point forward. By providing a greater understanding of the essential components of the fusogenic machinery, research can then focus on accessory proteins that shape the release properties that distinguish individual synapses, which ultimately will advance our understanding and of different neurotransmitter systems and neural networks.

4.1 Evolutionary conservation of SNAREs in vesicular trafficking and fusion

The ability to transport material between networks of highly organized, but also highly segregated intracellular compartments, or membrane-bound organelles, is a hallmark of eukaryotic cells. In such cells, the processes of vesicular trafficking and membrane fusion is fundamental to survival by mediating a multitude of homeostatic functions, such as secretion, endocytosis, intracellular digestion in lysosomes, and cell division (for review, see Hong 2005). The highly conserved superfamily of SNARE proteins, of which approximately 150 members have been discovered, mediates the vesicle targeting and fusion required for critical cellular processes in species throughout the four eukaryotic kingdoms of plants, fungi,

protists, and animals (Kloepper et al., 2007). Throughout evolution, preservation of specialized assemblies, or complexes, of SNAREs has been adapted to carry out membrane fusion within specific stages of distinct vesicular trafficking pathways (Fig. 4.1; see Weimbs et al., 2001). To this point, we propose that a specialized SNARE complex emerged, consisting of SNAP-25, syntaxin 1a, and VAMP-2, that is fundamental to regulated neurotransmission.

4.2 Evidence for a conserved universal neural SNARE complex

While the exact mechanisms have not been fully elucidated, a clear requirement for the calcium-triggered secretion of several diverse neurotransmitters and neuropeptides is the vesicular priming and subsequent membrane fusion facilitated by a heterotrimeric SNARE complex comprised of SNAP-25, syntaxin 1a, and VAMP-2. The function of these three proteins in the neurotransmission was revealed as the SNAREs were identified as specific targets for several potent neurotoxins, including Botulinum neurotoxins (BoNTs), whose mechanism of action was carried out through the site-directed cleavage of these proteins (for review, see Rossetto et al., 2006). In particular, studies using the SNAP-25-specific neurotoxin serotypes BoNT/A and /E, have shown that the calcium-triggered release of glutamate, acetylcholine, serotonin, dopamine, glycine, epinephrine, norepinephrine, insulin, prolactin, adrenocorticotropic hormone, and substance P is completely blocked (Blasi et al., 1993; Sadoul et al., 1995; Lawrence et al., 1996; Aguado et al., 1997; Masumoto et al., 1997; Raber et al., 1997; Washbourne et al., 1998; Steffensen et al., 1999; Welch et al., 2000;

Bergquist et al., 2002; Keller et al., 2004). In agreement with these findings, investigations by our lab and others have shown that in a *Snap25*-null mouse, action potential (AP)-dependent release of glutamate, acetylcholine, and catecholamines is absent in mutant neurons and adrenal chromaffin cells (Washbourne et al., 2002; Sorensen et al., 2003).

Further evidence to support a universal SNARE complex comprised of SNAP-25, syntaxin 1a, and VAMP-2 is the expression of functionally similar orthologs in several diverse species. An extensive search within selected eukaryotic genome libraries reveals highly homologous gene and protein sequences, as reflected by "percent identity" scores greater than 90%, to each of these three SNAREs in yeast, chickens, zebrafish, frogs and mammals (including humans), indicating that these proteins are highly conserved, presumably because of their requisite and specialized role in the fundamental process of regulated vesicle fusion (data acquired from individual PubMed Homologene [www.pubmed.gov] searches using the keywords: "SNAP-25", "Syntaxin 1A", and "VAMP-2").

Taken together, these findings suggest that, throughout evolution, eukaryotes have maintained a universal core complex, comprised of SNAP-25, syntaxin 1a, and VAMP-2, that functions as the basic fusogenic machinery necessary to carry out regulated secretion. The advantage of conserving a refined mechanism for calcium-triggered secretion is that this highly regulated process remains

functionally intact among species and cell types. However, because different isoforms of these fundamental SNAREs can be expressed, in combination with their post-translational modifications, such as phosphorylation, the universal SNARE complex has the potential to be a remarkably dynamic and flexible scaffold that dictates the assembly of modulating proteins, thus creating the diversity of release properties observed between species and cell types.

4.3 Extending the universal requirement for a SNAP-25-containing SNARE complex to AP-dependent GABAergic transmission

To test the model that SNAP-25 is part of a fundamental SNARE core complex universally required for calcium-triggered neurotransmission, our study investigated the expression and function of this t-SNARE in GABAergic neurons. The results of this research, described at length in the previous chapters, provide compelling evidence, at both the mRNA and protein level, of robust SNAP-25 expression in both fetal and adult central inhibitory neurons. Furthermore, detection of SNAP-25 in the presynaptic terminals of immature and mature GABAergic neurons indicated that appropriate transport and localization occurs persistently in these cells, inferring a functional role that is maintained into adulthood. In addition, by both a direct and indirect measurements of synaptic activity in SNAP-25-deficient cells, we show selective abrogation of AP-dependent neurotransmission, not AP-independent vesicular fusion, in GABAergic neurons.

Since our investigation, several labs have published findings that strengthen the data presented in the previous chapters. Using similar styryl dye assays and electrophysiological recording methods in *Snap25*$^{-/-}$ mice, two separate groups have confirmed that GABAergic neurons are dependent on SNAP-25 expression for evoked neurotransmission (Bronk et al., 2007; Delgado-Martinez et al., 2007). Therefore, we provided the first key evidence of the essential role that SNAP-25-containing SNARE complexes play in the evoked secretion of GABA, and thus, our results strengthen a proposed central role for this core fusogenic machinery in the calcium-triggered release of all neurotransmitters.

4.4 Challenging the model of a universal neural SNARE complex

The findings described in the previous chapters provide compelling evidence that has been corroborated by other research groups, however, results have been published that challenge the model of a universal neural SNARE complex required for regulated neurotransmitter release, especially in GABAergic transmission. To briefly summarize this debate, which is discussed extensively in Chapter 2, Matteoli and colleagues show that SNAP-25 is not significantly expressed in GABAergic neurons and that stimulated vesicular recycling at these inhibitory synapses is not blocked during BoNT/A and BoNT/E intoxication (Verderio et al., 2004; Frassoni et al., 2005). These results would suggest, therefore, that a SNAP-25-independent SNARE complex is utilized for calcium-triggered neuroexocytosis at GABAergic terminals, presumably containing the closely related SNARE, with SNAP-23 as the most likely alternative.

The protein SNAP-23 is a homologue of SNAP-25 and contains many of the same structural characteristics needed to interact with cognate SNAREs to form core complexes that drive membrane fusion. Like SNAP-25, SNAP-23 contains two SNARE motifs joined by a linker region that includes 5 cysteine residues that can be palmitoylated (Yang et al., 1999). However, despite structural similarities that may point to SNAP-23 as likely replacement for SNAP-25, this ubiquitously expressed protein was not able to fully rescue AP-dependent neurotransmission in *Snap25*$^{-/-}$ GABAergic neurons, but instead, appeared best suited for reversing minor alterations in constitutive miniature release (Delgado-Martinez et al., 2007). Interestingly, through the use of immunofluorescence, a recent report by Bragina and colleagues (2007) supports the Matteoli lab findings by showing a lack of SNAP-25 immunoreactivity in VGAT-positive synapses. Although, the results also show that SNAP-23 immunoreactivity, and hence, expression occurs extensively in glutamatergic synapses, but is only found in about 20% of GABAergic terminals (Bragina et al., 2007). In all, these results argue against SNAP-23 participation in a fully functional neural SNARE complex, and instead maintain that SNAP-23 is best equipped to serve its established role as part of a SNARE complex that carries out general, constitutive secretion in both neuronal and non-neuronal cells. Incidentally, to determine whether differences in the interpretation of fluorescent immunohistochemistry results were due to the specificity of anti-SNAP-25 antibodies selected by both the Matteoli group and our lab, we carried out additional experiments to compare these reagents and

these results are included as supplemental data in the appendix of this dissertation (Chapter 5).

4.5 Implications of dissecting the exocytotic machinery specific to different neurotransmitter systems

A full characterization of the exocytotic machinery is necessary to fully understand the interplay of the diverse circuitry within the brain and the integration of complex signaling during development as well as in mature networks. In addition, elucidating the specialized molecular foundation that sculpts the electrophysiological properties characteristic to synapse of a specific neurochemical phenotype could provide insight into both normal and pathological mechanisms. For example, an important process during development is the establishment of a balance between excitatory and inhibitory influences (E/I balance), which is tied to the regulated release of specific neurotransmitters, glutamate and GABA, respectively (Akerman and Cline, 2007). Disturbances in this process result in an inappropriate predominance of signaling by one of the neurotransmitters over the other leading to various pathological conditions such as epilepsy. Interestingly, SNAP-25, in particular, has been directly linked to several prevalent disorders in which disturbances of the E/I balance are suspected, such as schizophrenia (Thompson et al., 2003a; Thompson et al., 2003b), attention deficit hyperactivity disorder (ADHD; Brophy et al., 2002; Mill et al., 2002), and mood disorders (Fatemi et al., 2001). Furthermore, a coloboma mutant mouse strain, which possesses a hemizygous deletion of several genes

from chromosome 2q, including SNAP-25, displays a spontaneously hyperactive phenotype that is responsive to dextroamphetamine, an active ingredient in the widely used ADHD drug known as Adderall (Hess et al., 1995; Wilson 2000).

Based on previous findings showing differential expression within the brain and distinct functional characteristics of SNAP-25 isoforms (Bark et al., 1995; Boschert et al., 1996; Jacobsson et al., 1996; Jacobsson et al., 1999a; Jacobsson et al., 1999b; Sorensen et al., 2003; Bark et al., 2003; Delgado-Martinez et al., 2007), we originally proposed that the predominant expression of SNAP-25a is maintained in mature GABAergic neurons, functioning as a component of a unique molecular scaffold underlying the assembly of the exocytotic machinery that drives the sculpting of signature synaptic activity and characteristic electrophysiological properties ascribed to these cells (reviewed in Jonas et al., 2004). However, in contrast to our hypothesis, the evidence provided from experiments described in Chapter 3 demonstrate that GABAergic neurons undergo stereotypical developmental regulation of isoform expression much like that seen globally in the adult brain, resulting in greater relative levels of SNAP-25b transcripts (Bark et al., 1995). Although these findings were not ultimately supportive of our proposal, they did provide novel data that further characterized SNAP-25 expression in GABAergic neurons and glutamatergic neurons, thus continuing to extend the concept of this t-SNARE, specifically in the form of 25b, as an indispensable component of a universal neural fusogenic

core complex utilized in the fast, point-to-point transmission of at least two major classical neurotransmitter systems.

4.6 Dynamic modulation of presynaptic release machinery that could result in physiological differences between distinct synapses

Evidence for the role of SNAP-25 as an integral component of a universal SNARE complex required for calcium-triggered transmission comes from findings in various neurotransmitter systems. However, distinctive activity patterns, such as those found between GABAergic and glutamatergic neurons, provide a synapse with the abilities to operate with the speed, reliability, and precision demanded by the neural networks with which they integrate (reviewed by Jonas et al., 2004). Therefore, although the neural SNARE complex is a fundamental scaffold commonly utilized in classical neurotransmitter systems, it possesses the dynamic flexibility and versatility to recruit an array of binding partners to provide synaptic individualization. Allosteric regulation of presynaptic proteins via phosphorylation as well as differential expression of their isoforms, suggests that specialization of exocytotic machinery occurs through a cascade of protein-protein interactions individualized to a particular synapse.

Differential isoform expression of presynaptic modulatory proteins

As mentioned previously, several presynaptic proteins, specialized for the tight spatial and temporal regulation of neurotransmitter release, are differentially

expressed between inhibitory and excitatory synapses. Patterns of differential isoform expression within distinct synapses have been shown for several proteins involved in neuroexocytosis, such as Munc 13 (Augustin et al., 1999; Rosenmund et al., 2002; Varoqueaux et al., 2002), Rim1α (Rab3-interacting molecule 1α; Schoch et al., 2002), synapsins (Feng et al., 2002; Gitler et al., 2004; Kielland et al., 2006; Bragina et al., 2007), complexins (Yamada et al., 1999; Eastwood and Harrison, 2000), synaptogyrin (Belizaire et al., 2004), SV2 (Bajjalieh et al., 1994; Crowder et al., 1999), and synaptophysin (Fyske et al., 1993; Bragina et al., 2007). Furthermore, differences in isoform function within specific neurotransmitter systems, specifically glutamatergic and GABAergic neurons, have been shown for Munc13, RIM1α , and synapsin, indicating a modulatory role for these proteins that shape the physiological parameters governing neuronal activity.

For example, munc 13, which is expressed as three different isoforms, interacts with syntaxin 1a in the neural SNARE complex to promote neurotransmitter release and it can be functionally modulated through protein kinase C (PKC) phosphorylation (Betz et al., 1998) The isoforms of munc 13 are differentially expressed in GABAergic and glutamatergic synapses and that PKC-phosphorylation of munc 13-2 has differential effects when compared to munc 13-1, resulting in greater calcium sensitivity of neurotransmitter release as well as the ability to recruit a increased size of RRP (Augustin et al., 1999; Rosenmund et al., 2002; Varaquox et al., 2002). Furthermore, genetic deletion of the presynaptic protein RIM1α, which has been shown to interact with several

critical exocytotic proteins, such as munc 13, Rab3A, and synaptotagamin, provides opposite effects in paired-pulse facilitation and neurotransmitter release in GABAergic and glutamatergic neurons (Schoch et al., 2002).

As perhaps one of the most documented example, the vesicle protein synapsin is present as three isoforms which are differentially expressed throughout the brain (Kielland et al., 2006; Bragina et al., 2007). Several reports have been published showing distinct differences in the function of each isoform within GABAergic and glutamatergic neurons. For instance, during prolonged depolarization, synapsin I deficient synaptosomes show decreased glutamate release with increased facilitation at excitatory synapses, and decreased transmission at inhibitory synapses (Rosahl et al., 1993; Rosahl et al., 1995; Lietal.,1995; Teradaetal.,1999). In contrast, genetic ablation of synapsin III decreases basal transmission at inhibitory synapses, but not excitatory synapses (Feng et al.,2002). Lastly, a triple-knockout of all synapsin isoforms revealed that while synapsins are responsible for the maintenance of the reserve pool of glutamatergic vesicles, their role in GABAergic neurons is to regulate the size of the RRP (Gitler et al., 2004).

Regulation of presynaptic protein interactions via phosphorylation

The ability of a standard SNARE complex to provide the basis for customized fusogenic assemblies involves regulated interactions with discrete proteins. Phosphorylation provides a mechanism for controlling individual SNARE interactions as well as tightly regulating membrane fusion. The amino acid sequence of SNAP-25 contains many prospective sites for such kinases to act; with three potential residues, serine 28 and 187 as well as threonine 29, as substrates for PKC (Genoud et al., 2004) and one side chain, threonine 138, that is likely targeted by PKA (Risinger and Bennett, 1999).

In studies comparing the regulatory effects of each kinase, it appears that PKA phosphorylation regulates the size of the RRP, whereas PKC controls the refilling of the vesicle pools after they have been depleted (Nagy et al., 2004). PKC-mediated phosphorylation of serine 187 has been shown to inhibit SNAP-25 binding to syntaxin 1a as well as shifting SNAP-25 localization from the cytosol to the presynaptic membrane (Shimazaki et al., 1996; Kataoka et al., 2000; Takahashi et al, 2003). In addition, casein kinase I and II-mediated phosphorylation of syntaxin 1a results in a conformational change to the “open” configuration, resulting in decreased affinity to munc 18-1 and increased ability to bind synaptotagamin, SNAP-25, and VAMP-2, resulting in increased levels of SNARE complex assembly and fusion (Risinger and Bennett, 1999; Foletti et al., 2000; Dubois et al., 2002). Therefore, direct phosphorylation of SNAREs

provides explicit control over neurotransmitter release through the regulation of the kinetics in core complex assembly, providing another opportunity to shape synaptic properties to suit the requirements of distinct neurotransmitter systems.

In addition to kinases directly regulating the SNARE complex, neurotransmitter release can be further modified through phosphorylation of accessory proteins of the exocytotic machinery. The effects on neurotransmission have been extensively reported for a host of presynaptic proteins. For the sake of brevity, Figure 4.2 shows a table summarizing the effects of phosphorylation on a selection of proteins intimately involved in synaptic vesicle fusion (reviewed by Snyder et al., 2006).

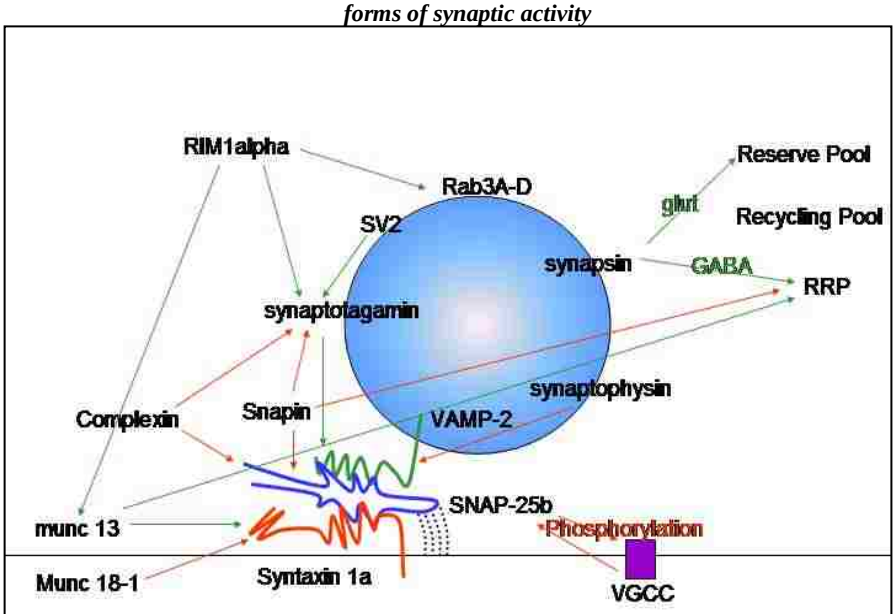
Figure 4.2 Modulation of the presynaptic release machinery via phosphorylation (reproduced from Snyder et al., 2006)

Phosphorylation of Known Accessory SNARE Proteins

Protein	Kinase	AA site(s)	Action
Munc-18	PKC cdk5	306, 313 574	Disassociation from syx Minor action to disassociate from syx
Synaptotagmin	CKI CaMKII and PKC	128 or 129 112	May alter calcium binding. Increases binding to syx
NSF	PKC	237	Decreases interaction with SNAREs
Snapin	PKA TKMET	50	Interacts with SNAP25, increases interaction of synaptotagmin with SNAREs and decreases readily releasable pool
Synaptophysin	CaMKII	?	May affect channel activity
N-type calcium channels	PKC CaMKII	? (α_1B subunit)	Inhibits binding of syx 1A and SNAP25
P/Q-type calcium channels	cdk5	724, 981	Inhibits SNARE interaction and neurotransmitter release

In summary, at the physiological level, the various protein interactions described above could act as molecular switches that participate in the dynamic interactions that facilitate vesicle fusion (Fig. 4.3). In addition, specific functional differences in excitatory and inhibitory synapses, indicate that presynaptic modulators play unique roles at distinct synapses. Globally, in addition to presynaptic modulation, specialization of individual synapses that are present within the dendrites of postsynaptic cells optimize the detection of the neurochemical signal released by a particular terminal (for review, see Lardi-Studler and Fritschy, 2007).

Figure 4.3 Differential interactions of presynaptic proteins that underlie distinct forms of synaptic activity



Reported interactions between proteins involved in neurotransmitter release. Red arrows indicate inhibition and green arrows indicate enhancement of release.

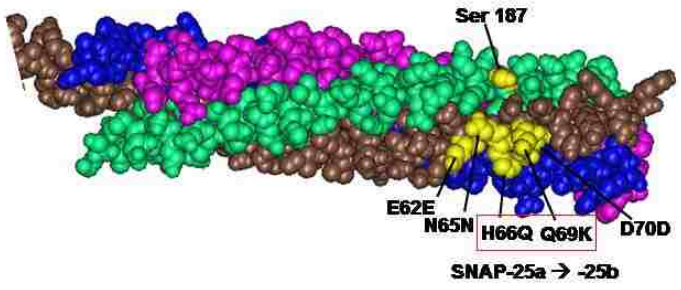
4.7 Differential SNAP-25 isoform expression in neuroendocrine cells and how functional differences might contribute to fusion mechanisms for synaptic vesicles and large dense core vesicles

Although we were able to confirm the expression and function of SNAP-25b in the major excitatory and inhibitory central neurons, we were not able to identify specific cells that maintain predominant SNAP-25a expression into adulthood. It is well documented that several distinct nuclei of neuronal populations, namely neurosecretory cells largely involved in the release of peptides and hormones, retain SNAP-25a (Bark and Wilson, 2004). More specifically, SNAP-25a can be easily detected in the cells of the pituitary, hypothalamus, and adrenal chromaffin cells, as well as pancreatic beta cells (Jacobsson and Meister, 1996; Jacobsson et al., 1999b) (Jacobsson et al., 1994; Gonelle-Gispert et al., 1999; Grant et al., 1999), which suggests that these cells depend on a release mechanism, or components of the exocytotic machinery, specific for the fusion of large dense core vesicles (LDCVs).

This is further confirmed by results showing that activity-dependent increases in SNAP-25 expression result in the preferential selection of the isoform naturally found in the cell, such that 25b is found in stimulated dentate gyrus granule cells (Hepp et al., 2001) and 25a in neurosecretory magnocellular hypothalamic neurons (Jacobbson et al., 1999b). In addition, the specificity of isoform function in neuroendocrine cells has been demonstrated in *Snap25* knockout adrenal chromaffin cells, where rescue with 25a fully restored evoked catecholamine

release, however, when 25b was added, the number of vesicles recruited to the RRP was abnormally large, resulting in the inappropriate “over-rescue” of these neuroendocrine cells (Sorensen et al., 2003; Nagy 2005). Additionally, when performing the same experiment on cultured hippocampal *Snap25*$^{-/-}$ neurons, unlike 25b, 25a was not able to recruit as large a RRP to the synapse and only partially restored synaptic function (Delgado-Martinez et al., 2007). Therefore, the exclusive expression of a particular SNAP-25 isoform may provide the necessary scaffold needed to recruit and assemble the optimal exocytotic machinery necessary for different modes of vesicular fusion.

Figure 4.4 Prospective binding pocket differentiall phosphorylated in SNAP-25 isoforms



Model of the four-barreled SNARE complex with H66K, Q69K, and Serine 187 highlighted (yellow) to show spatial organization of a prospective binding pocket. Adapted from Sutton et al., 1998.

Several differences that distinguish the two SNAP-25 isoforms may also provide the basis for their involvement in different types of membrane fusion. Firstly, the SNAP-25 isoforms differ in only nine amino acids, however, the difference in sequence includes the rearrangement of the four cysteine residues that are palmitoylated, resulting in differential subcellular localization for SNAP-25a (cell body and axons) versus 25b (presynaptic terminals), suggesting

different sites of function in the cell (Oyler et al., 1991; Bark et al., 1995; Andersson et al., 2000). Perhaps most interesting is the differential ability for each isoform to be phosphorylated, resulting in differential regulation of calcium dynamics at the synapse (Gonelle-Gispert et al., 2002; Verderio et al., 2004; Pozzi et al., 2008; Kashaul and Wilson, unpublished results from our lab). The site of PKC-phosphorylation is identical in both isoforms, residing at a 187-ser residue of the second, or C-terminal SNARE motif/coil, therefore, it is expected that 25a and 25b would display an identical ability to be phosphorylated (Kataoka et al., 2000). However, the answer may lie in 2 of the 9 residue substitutions, H66Q and Q69K, which have been shown to be the basis of the functional differences between 25a and 25b (Nagy et al., 2005). These amino acid substitutions occur in the N-terminal SNARE domain, but interestingly enough, when assembled in the full four-barreled SNARE complex, the side chains are exposed via external rotation and appear to align with the serine 187 residue (Fig. 4.4). Therefore, the charge differences found between isoforms, combined with the presence or absence of phosphorylation, may result in a customizable binding pocket within the SNARE complex that allows for the differential recruitment of proteins necessary for either fast, point-to-point neurotransmission (via a SNARE complex including SNAP-25b) or regulated secretion of neuroendocrine cells (utilizing a SNAP-25a-containing scaffold).

4.8 Limitations of the current study

The experimental methods used to test our hypothesis provided valuable evidence that supports SNAP-25 expression and function in GABAergic neurons. However, like all investigations, there are certain limitations inherent to the methods used and the samples available. For example, in the previous reports by the Matteoli group, fetal cultured hippocampal GABAergic neurons displayed a transient expression of SNAP-25 that is dramatically decreased around DIV2, leading to a reduction in protein levels that ultimately fall below the level of detection at DIV10 (Frassoni et al., 2005). In addition, this group showed that SNAP-25 immunoreactivity is not found within GABAergic terminals of the adult brain, suggesting a transient expression and function that does not persist in mature GABAergic neurons (Verderio et al., 2004). In contrast, our results demonstrated that colocalization of robust and punctate immunostaining patterns for SNAP-25 and two independent GABAergic markers occurs in fetal hippocampal neurons and remains so through DIV21, as well as expression of this t-SNARE in mature central inhibitory neurons in several regions of the brain (Fig 2.5I). However, the functional evidence provided here, while supporting results in other independent studies (Bronk et al., 2007; Delgado-Martinez et al., 2007), are limited to SNAP-25 function that occurs in fetal neurons and does not address a role for this t-SNARE in neuroexocyosis within mature *Snap25*$^{-/-}$ synapses. Although normal brain development occurs in utero, the lack of neurotransmitter release in SNAP-25 deficient mutant mice results in perinatal

death via paralysis of the diaphragm. Therefore, we cannot eliminate the possibility of a transient role of SNAP-25 function in GABAergic neurons.

However, the persistence of SNAP-25 expression within presynaptic terminals of both fetal and mature GABAergic neurons provides compelling evidence to indicate continued function throughout development of inhibitory neurons. Directed localization and spatial organization of cognate SNAREs in target subcellular compartments speaks largely to their functional roles by virtue of the SNARE hypothesis. The SNARE hypothesis, originally advanced by Sollner and colleagues (1993), states that a functional fusogenic core complex must be assembled through interactions between proteins situated on opposing membranes. Therefore, this would suggest that identifying areas in which a specific SNARE, such as SNAP-25, is highly concentrated infers that this protein has a potential functional role in the specialized intracellular trafficking pathways and/or subsequent membrane fusion events of that area.

In order to prevent ectopic assembly of the obligate fusogenic machinery for calcium-triggered neurotransmitter release, spatial regulation of cognate SNAREs is carried out through post-translational modifications or binding to inhibitory proteins, resulting in the prevention of energetically-favored interactions outside of the presynaptic terminal compartment (reviewed in Snyder et al., 2006). One example is the dynamic regulation of syntaxin 1a, which contains a

specialized N-terminal domain that spontaneously self-associates, resulting in a non-reactive, “closed” conformation that is recognized and stabilized by munc 18-1, an inhibitor of neuroexocytosis (Dulubova et al., 1999; Misura et al., 2000; Yang et al., 2000; Perez-Branguli et al., 2002). This mechanism shields the SNARE region from potential non-specific interactions during transport to the presynaptic membrane, thereby sequestering the highly-reactive helical motif until directed core complex assembly occurs. The synaptic vesicle protein synaptophysin performs a role analogous to munc 18-1 by binding to VAMP-2 and preventing interactions with other SNAREs until complex assembly and vesicular fusion is imminent (Calakos and Scheller, 1994; Edelmann et al., 1995; Becher et al., 1999; Valtorta et al., 2004; Bonanomi et al., 2005).

SNAP-25 is no exception to spatial and temporal control as several levels of regulation exist for this t-SNARE. Like other SNARE proteins, in order to induce vesicular fusion, SNAP-25 must be membrane associated. However, unlike the cognate proteins syntaxin 1a and VAMP-2, which are each embedded into the membrane via a single C-terminal transmembrane domain, cytosolic SNAP-25 requires palmitoylation, a posttranslational modification occurring at four centrally located cysteine residues, in order to be anchored to the target membrane (Hess et al., 1992; Veit et al., 1996; Gonzalo et al., 1999; Koticha et al., 1999; Washbourne et al., 2001; Loranger et al., 2002). Palmitoylation of SNAP-25 requires all four cysteine residues within the context of a specific stretch of amino

acid sequence to the extent that a mutation in a single cysteine side chain will dramatically reduce the ability of this t-SNARE to anchor to the membrane (Lane and Liu, 1997; Gonzalo et al., 1999; Gonelle-Gispert et al., 2000). In addition, results from our lab and others demonstrated that palmitoylation of SNAP-25 not only allows for membrane association, but can also contribute to SNARE protein interactions and direct localization to specific lipid rafts, or domains, integrated within the synaptic membrane (Washbourne et al., 2001; Kang et al., 2004; Salaun et al., 2004; Salaun et al., 2005). Therefore, due to the multiple levels of regulation ensuring the proper trafficking, localization, and concentration of specific SNAREs within subcellular compartments reserved for membrane fusion, the results of my initial immunofluoresence experiments, which verified the presence of SNAP-25 within GABAergic neurons, and more specifically, presynaptic terminals, strongly indicated a functional role for this t-SNARE in the neurosecretion of GABA. Moreover, the individual colocalization of the two different vesicular transporters with SNAP-25, but not with each other, substantiates the expression of SNAP-25 in the presynaptic boutons of GABAergic and glutamatergic neurons in fetal hippocampal cultures that persists through adulthood.

4.9 Future Studies

In addition to establishing SNAP-25 expression in GABAergic neurons, the findings described in the previous chapters provide convincing results showing the persistent expression and directed targeting of this t-SNARE to presynaptic

terminals, indicating a functional role in inhibitory neuroexocyosis. Furthermore, we show evidence that extends to GABAergic neurons the universal requirement for SNAP-25-containing SNARE complexes in calcium-triggered neurotransmission and confirm that, as in other neurotransmitter systems, constitutive, AP-independent release persists in the absence of this t-SNARE. While the results shown here have provided valuable insight into the molecular machinery behind evoked GABA release, future studies could be conducted that would continue to focus on dissecting the elaborate fusogenic apparatus specialized for both the slow fusion of LDCVs as well as the fast, point-to-point transmission carried out by synaptic vesicle recycling. Concentrating on SNAP-25 isoforms, a thorough characterization of persistent SNAP-25a expressing cell types within the adult brain as well as the neurochemicals they release would begin to shed light on the differential use of this t-SNARE. In addition, initial investigations could explore the existence of a phosphorylated “binding pocket” in SNARE complexes containing different SNAP-25 isoforms. The results of this experiment could reveal a novel target for experimental and/or therapeutic manipulation of neuropeptide and neurotransmitter secretion via small binding molecules.

Finally, in order to confirm the persist functional significance of SNAP-25 in mature GABAergic neurons as well as other neurons of the adult brain, a conditional knockout mouse that allows for targeted deletion of this t-SNARE in

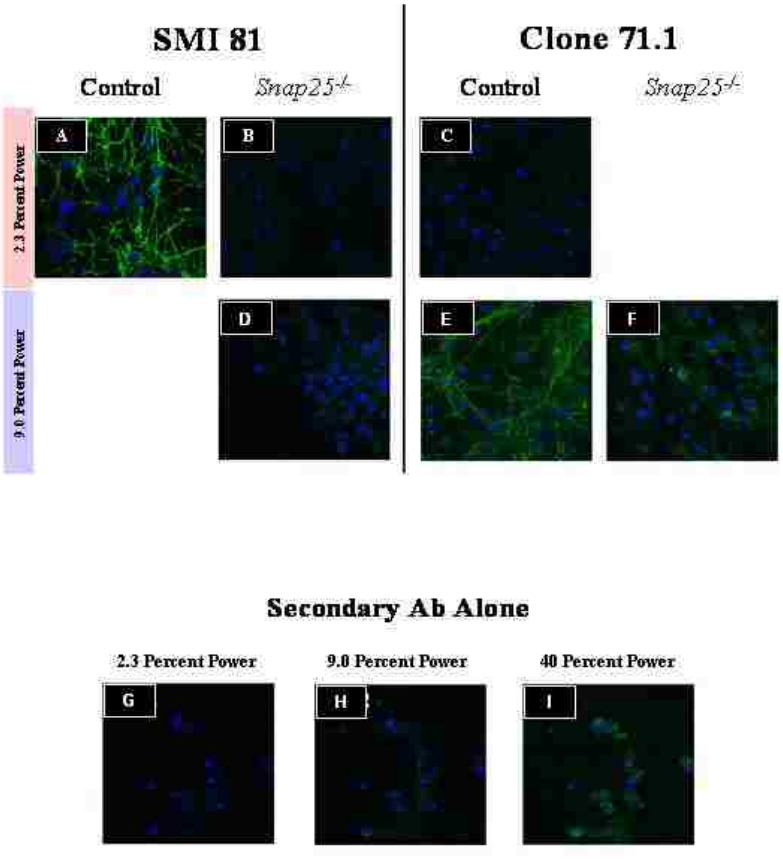
defined brain regions will be necessary to extend these findings into adulthood. A mutant strain carrying a floxed SNAP-25 gene is currently being developed in our lab and can be used for such future studies. Furthermore, elucidation of accessory protein interactions with SNARE complexes and the subsequent assembly of specialized fusogenic machinery, especially in GABAergic and glutamatergic transmission, will yield valuable knowledge in the biogenesis of the E/I balance and identification of specific defects in neural activity that lead to related neuropathologies.

5. Appendix

5.1 Fluorescent immunohistochemistry comparing the binding specifificity of two different SNAP-25 antibodies

To evaluate the possibility that differences in antibody specificity were responsible for the discrepancies between our results and those published previously, I compared the anti-SNAP-25 monoclonal antibody SMI 81 I used in past experiments (Tafoya et al., 2006; Sternberger Monoclonals) with clone 71.1, the antibody used in other reports (Verderio et al., 2004; Frassoni et al., 2005; Synaptic Systems).

Figure 5.1 Comparison of SNAP-25 antibody specificity



Immunostaining of cultured hippocampal neurons with two separate SNAP-25 monoclonal antibodies reveals alternative patterns of reactivity. Cultured hippocampal neurons were probed with separate SNAP-25 monoclonal antibodies SMI 81 (green in panels *A, B, and D*) and clone 71.1 (green in panels *C, E, and* F). Results using a secondary antibody alone are shown in panels *G-I*.

Using an identical protocol as described in Chapter 2 (Materials and Methods; Tafoya et al., 2006), E17.5 *Snap25* null mutant and control hippocampal neuronal cultures were probed with either SMI 81 or 71.1 antibodies (Fig. 5.1, green) followed by cross-staining of the nuclei with ToPro3 (Fig. 5.1, blue). Employing confocal microscopy, I visualized the samples and observed several differences between immunostaining patterns of each antibody. SMI 81 immunoreactivity in control neurons was consistent with the expected localization of SNAP-25 wherein there is robust punctate immunoreactivity throughout the field, presumably within presynaptic terminals, as well as more continuous staining throughout the neurites, probably reflecting intracellular transport of the t-SNARE along axons (Fig. 5.1A). However, control cultures immunolabeled with 71.1 showed no detectable signal at the laser settings used for SMI 81 detection (2.3 percent power; Fig. 5.1C) and was only evident at a higher laser power level (9.0 percent power; Fig. 5.1E). At this higher laser power setting, unlike SMI 81, clone 71.1 immunoreactivity displayed a more diffuse staining pattern with less focal, demarcated punctate staining and less overall neurite staining, which surprisingly appeared most abundantly throughout the soma (Fig. 5.1E).

Next, as a negative control, I examined *Snap25*$^{-/-}$ hippocampal cultures probed with each antibody in order to determine whether non-specific binding occurs. SNAP-25 knockout cultures incubated with SMI 81 showed no detectable signal at either the low (Fig. 5.1B) or higher laser power settings (Fig. 5.1D). However,

when probing with 71.1, robust immunoreactivity was apparent within the soma, especially around the nucleus (Fig. 5.1F). Control experiments were performed using only the secondary anti-mouse Alexa488-conjugated antibody to ensure that this finding did not result from non-specific binding of the secondary antibody. As expected, immunoreactivity was not detected in these samples, and visible levels of signal were only evident at a very high laser power setting, presumably from background autofluorescence inherent to the cells (Fig. 5.1G-I).

Overall, these results raise some concerns about the specificity of the 71.1 antibody in immunohistochemical analysis of SNAP-25 expression in GABAergic neurons. Based on this and the experiments described in Chapter 2 (Tafoya et al., 2006), the SMI 81 antibody shows no SNAP-25 reactivity in the soma of control neurons at any point from DIV9-21 and no cross-reactivity to other proteins when used for Western blots of SNAP-25 knockout neuronal extracts (Fig. 2.1 and 2.6). Interestingly, previous reports showed that SNAP-25 expression (based on 71.1 immunoreactivity) was mainly in the perinuclear/Golgi region of cultured GABAergic neurons and not localized to the presynaptic terminals at later time points (Frassoni et al., 2005). This is similar to our results as robust perinuclear staining was readily detected and appeared greater than the reactivity in neurites of wild type neurons. However, and more importantly, this perinuclear stain persisted in SNAP-25 knockout mutant neurons, which indicates that 71.1 antibody reacts with a related epitope, distinct from SNAP-25.

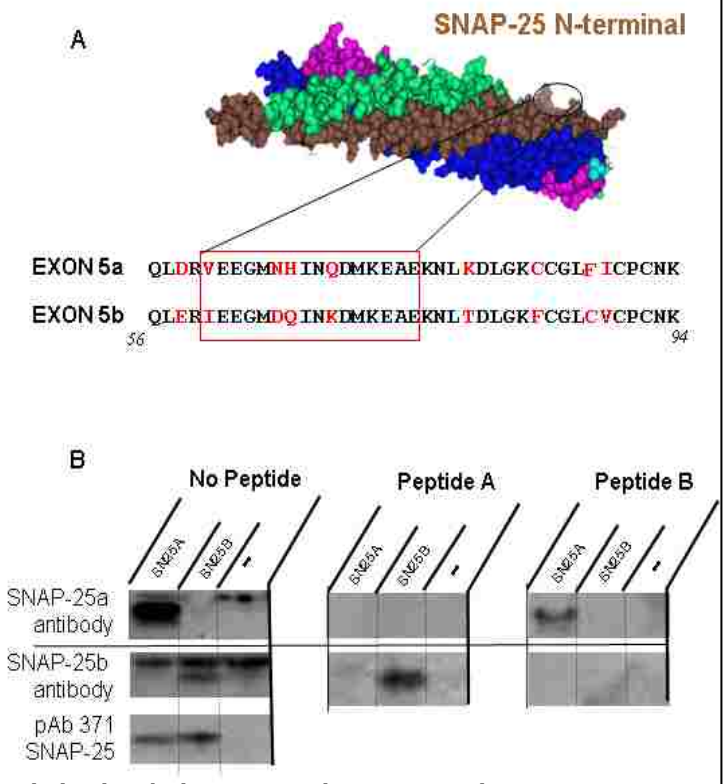
In addition, the somatic staining was so robust compared to neurite staining, that when I adjusted the microscope settings to prevent signal saturation, the synaptic staining often would fall below detectable levels, therefore making it likely that specificity may have contributed to the differences seen between our fluorescent immunohistochemistry results (Tafoya et al., 2006) and those seen previously (Verderio et al., 2004; Frassoni et al., 2005).

5.2 Development of SNAP-25 isoform specific antibodies

Western blot application

I have developed a novel set of rabbit polyclonal antibodies that can distinguish between SNAP-25a and SNAP-25b protein. These antibodies were raised against synthetic peptides containing sequences (residues 60-75) that differ in four amino acids. This region was selected because it contains the amino acid substitutions that underlie the functional differences attributed to the isoforms in chromaffin cells (Nagy et al., 2005). Because these residues are accessible to the cytosol, recruitment of accessory proteins may differ between SNAP-25 isoforms, leading to their differential function. Moreover, the side chains of these distinctive residues are exposed to the exterior of the four-barrel SNARE

Figure 5.2: Specificity of SNAP-25 isoform antibodies

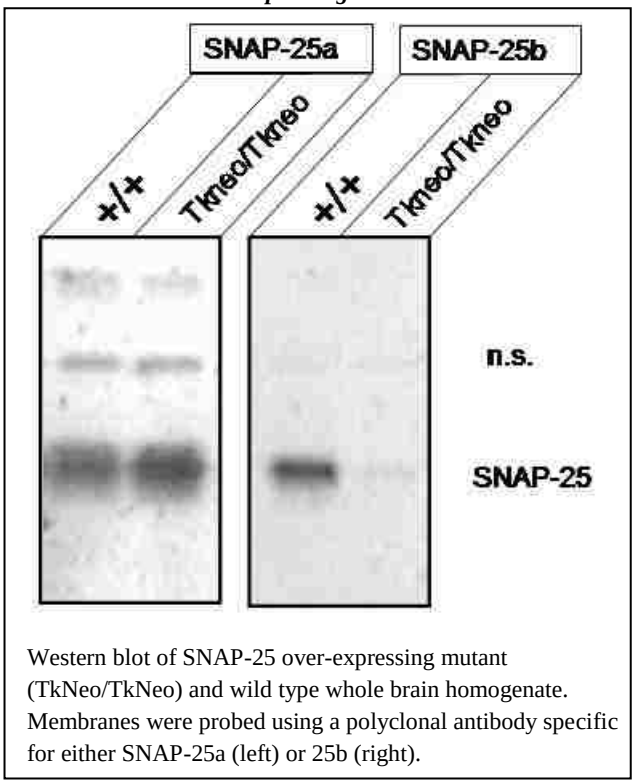


Polyclonal antibodies were raised against a peptide sequence containing four amino acid differences found between SNAP-25 isoforms. This region of SNAP-25 is highlighted in a space-fill model of the four-barrel helix of the neural SNARE complex (Adapted from Sutton et al., 1998). (A). Western blotting of isoform specific antibodies show robust immunoreactivity in appropriate transfected Cos 7 cell extracts that is blocked when the antibody is preblocked with its respective immunogenic peptide (B). Transfection of cells was confirmed using a polyclonal antibody (371) raised against a sequence common to both SNAP-25 isoforms.

complex, potentially enabling us to detect the isoforms when inserted in an assembled SNARE complex (Fig. 5.2A).

These antibodies show specific immunoreactivity to their respective isoform in western blots of transfected Cos7 cell protein extracts. Importantly, the detection of the specific 25 kDa isoform protein was abolished when the antibody was preblocked by incubation with the immunizing peptide, thus demonstrating isoform specificity of our peptide directed antibodies (Fig. 5.2B). In addition, probing whole brain homogenate prepared from SNAP-25a over-expressing mutant (designated TkNeo/TkNeo) with anti-SNAP-25a antibody resulted in a 25 kDa band with greater signal intensity than the wild type control sample (Fig. 5.3, left). In contrast, there was very little reactivity in lanes containing mutant samples the anti-SNAP-25b isoform antibody was used (Fig. 5.3, right).

Figure 5.3 Isoform antibody specificity in SNAP-25a overexpressing mutants

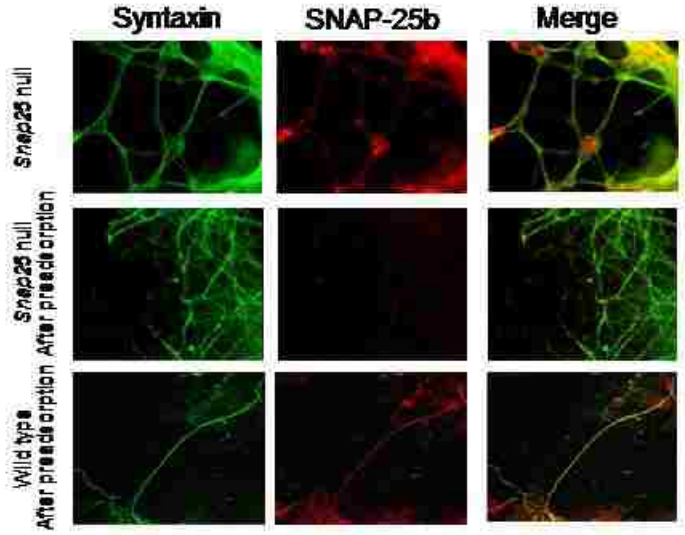


Western blot of SNAP-25 over-expressing mutant (TkNeo/TkNeo) and wild type whole brain homogenate. Membranes were probed using a polyclonal antibody specific for either SNAP-25a (left) or 25b (right).

Application of SNAP-25 isoform antibodies in fluorescent immunohistochemistry

While these antibodies reliably detect SNAP-25 isoforms in western blot analysis, they also detect several non-specific bands, precluding their use in immunohistochemistry. Thus far, our attempts to purify the antibodies further to eliminate these non-specific signals have been somewhat successful (Fig. 5.4). Nevertheless, with further preadsorption and possibly positive selection strategies it may be possible to arrive at isoform specific antibodies necessary to resolve the differential expression of SNAP-25 in GABAergic and other specific neurons in adult brain. In addition, the use of these antibodies in applications such as immunoprecipitation can yield valuable insight into the proteins associated with isoform-specific SNARE complexes underpinning differential transmission in the complex neurocircuitry of the brain.

Figure 5.4 Fluorescent immunohistochemistry of Snap25$^{-/-}$ and wild type neurons



Immunostaining of DIV12 cultured hippocampal neurons with SNAP-25b specific antibody. Before adsorption, immunoreactivity is found in SNAP-25 deficient cells (top row, red). After preadsorption, immunoreactivity is absent in mutant cultures (middle row), but is detectable (bottom row, red) and colocalizes with syntaxin (green) in wild type samples.

5.3 Spontaneous AP-independent vesicular recycling in GABAergic and glutamatergic presynaptic terminals in the absence of SNAP-25

In this study, unpublished supplemental data for Tafoya et al. (2008), I investigated the effects of SNAP-25 ablation on spontaneous neuronal activity in cultured GABAergic and glutamatergic hippocampal neurons. Results from the above referenced manuscript, as reproduced in Chapter 3 of this dissertation, demonstrated that SNAP-25 deficient neurons lacked calcium oscillations propagated by AP-dependent synaptic transmission, but exhibited increased resting intracellular calcium ($[Ca^{2+}]_i$) compared to control neurons. Increased $[Ca^{2+}]_i$ is known to enhance the frequency and amplitude of spontaneous synaptic activity, as measured by postsynaptic currents (PSCs or minis; for review, see Collin et al., 2005). However, despite a higher resting $[Ca^{2+}]_i$, the frequencies of both AP-independent excitatory and inhibitory synaptic events (mEPSCs and mIPSCs, respectively) are substantially reduced in SNAP-25 deficient knockout neurons (Washbourne et al., 2002; Tafoya et al., 2006; Bronk et al., 2007; Delgado-Martinez et al., 2007).

Interestingly, the size of the readily releasable pool (RRP) of vesicles responsive to evoked stimuli is increased following prolonged inhibition of synaptic activity by either TTX block of action potentials (Murthy et al., 2001) or blockade of AMPA receptors (Thiagarajan et al., 2005), suggesting that the development of synaptic vesicle pools within presynaptic terminals may be subject to $[Ca^{2+}]_i$ mediated, activity-dependent homeostatic regulation. I, therefore, examined whether AP-

independent, constitutive vesicular recycling that supports spontaneous synaptic activity differed between the SNAP-25 deficient knockout neurons, lacking evoked synaptic activity, compared to wild type and heterozygote neurons treated acutely with TTX to block stimulus evoked synaptic responses.

To evaluate spontaneous vesicle recycling, I assayed uptake of an aldehyde–fixable analog of the styryl dye FM 1-43 (fixable analog of N-[3-triethylammoniumpropyl]-4-[4-[dibutylamino]styryl]pyridinium dibromide [FM1-43FX; Molecular Probes; Eugene, OR]), that is conventionally used in vesicle recycling studies (Betz and Bewick, 1992) in high density dissociated hippocampal cell cultures prepared as above from *Snap25*-null mutant and control (heterozygote and wild type) E17.5 fetuses at 10-12 DIV. The use of FM1-43FX permitted visualization of the co-localization of internalized dye within these terminals, after paraformaldehyde fixation, permeabilization and immunostaining for these vesicular reuptake transporters (Brumback et al., 2004; Tafoya et al., 2006). Previous studies had demonstrated that in media containing 5 mM Ca^{2+}, to enhance the frequency of spontaneous neuronal activity, a 20 minute incubation was sufficient to label the constitutive recycling pool in presynaptic terminals (Prange and Murphy, 1999). However, because this high extracellular calcium concentration would raise $[Ca^{2+}]_i$ and potentially offset the difference between the resting calcium levels of wild type and SNAP-25 knockout neurons, I used a longer loading protocol of a 60 minute incubation at 37°C in a

low potassium, modified Tyrode's buffer with the same lower, physiologically-relevant concentration of 2 mM Ca^{2+} (150 mM NaCl, 4 mM KCl, 2 mM MgCl2, 10 mM glucose, 10 mM HEPES, and 2 mM CaCl2, pH 7.4) as used in the calcium imaging experiments outlined in Chapter 3 (Tafoya et al., 2008). During this incubation and throughout the duration of the experiment, all buffers were used at 37°C and contained 10 µM CNQX, 25 µM APV, 20 µM bicuculline, and 0.6 µM tetrodotoxin (TTX) to prevent spontaneous action potential (AP)-dependent neuronal activity. Synaptic vesicles were loaded in the presence of 15 µM FM1-43FX for 60 min at 37°C, then cells were immediately washed with modified Tyrode's solution in the absence of dye for before fixation in a 4% paraformaldehyde (PFA)/4% sucrose solution for 5 min at room temperature.

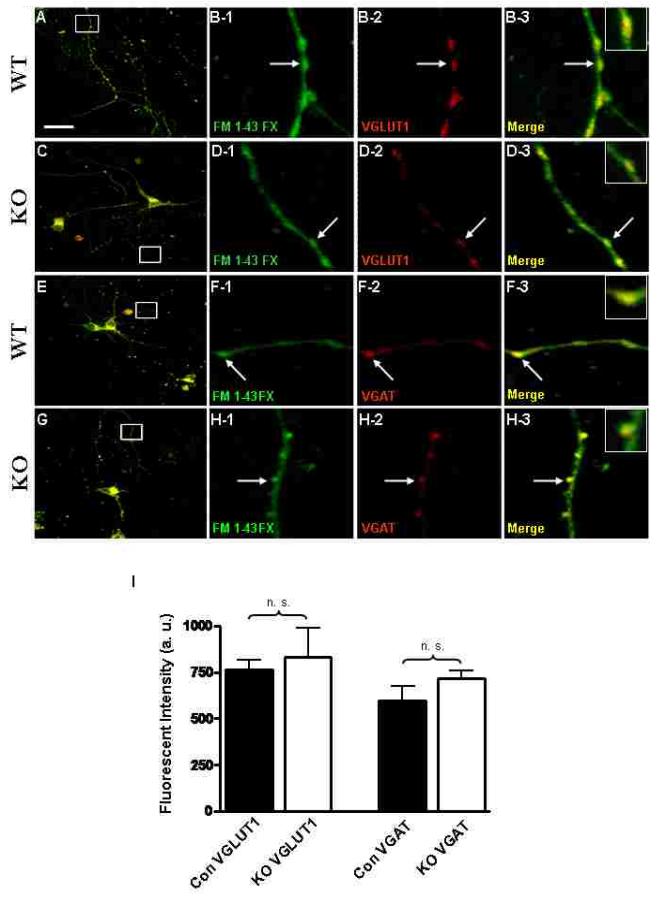
To focus on endocytosis of synaptic vesicles, I restricted our measurements of styryl dye uptake to glutamatergic and GABAergic terminals, defined by the expression of the vesicular transporters for glutamate (VGLUT1) or GABA (VGAT). The fixed cultures were immunostained with either anti-VGAT or -VGLUT1 monoclonal antibodies (Synaptic Systems, Goettingen, Germany) followed by Alexa 647-conjugated anti-mouse secondary antibody (Molecular Probes), and then visualized with a Zeiss LSM 510 META/Axiovert 100M laser confocal microscope using a 63X oil differential interference contrast (DIC) objective (optical slice, 0.81 µm; numerical aperture [N. A.], 1.4). META photodetectors were configured to recognize fluorescent emissions within the

spectral range of 556–716 nm, and the peak emissions of FM1-43FX (598 nm) and Alexa 647 (663 nm) were captured at 585–609 and 652–673 nm, respectively. Using the mean region of interest (ROI) intensity function of the LSM 510 bundled software, I measured FM1-43FX fluorescence intensity colocalized within the immunoreactive punctate staining of either VGAT or VGLUT1. A region of interest (ROI) was restricted to a single point of five pixels that overlay the majority of area centered within each puncta.

Using a single value per field, I calculated the average fluorescence intensity from approximately ten puncta. Three fields per animal were examined and averaged to obtain a final single value for synaptic activity in each animal that was used for statistical analysis (n = 3 animals). Using Wilcoxon signed rank test, data analysis was carried out following subtraction of background autofluorescence (Graphpad Prism 4 software, San Diego, CA). All values expressed as mean ± SD. Although brightness/contrast was adjusted for clarity in images used in figures (Adobe Photoshop 7.0 software; Adobe Systems Inc., San Jose, CA), all data for the experiment was collected before any such manipulations.

As shown in Figure 5.5, uptake of the FM 1-43FX dye (green) could be readily detected in control and knockout cultures after incubation in the presence of the receptor antagonists APV, CNQX, bicuculline, and the sodium channel blocker TTX. As expected, punctate FM dye fluorescence was found colocalized with compact focal areas of VGLUT1 immunoreactivity in control cultures (Fig 5.5A-B3), consistent with AP-independent

Figure 5.5 Action potential independent constitutive vesicle endocytosis persists in both glutamatergic and GABAergic



Hippocampal neurons (DIV 10-12), prepared from SNAP-25 knockout (KO) and wild type (WT) fetuses were loaded with 15 µM FM 1-43FX, in the presence of APV, CNQX, bicuculline, and TTX for 60 minutes at 37°C before immunostaining for VGLUT1. Panels A and C show representative laser confocal micrographs of dye uptake and VGLUT1 staining in wild type and *Snap25*$^{-/-}$ mutant neurons (scale bar, 50 µm). The following series of panels (B1–B3, D1–D3) represent digitally magnified (7X; scale bar, 7 µm) images of separate and merged color channels demonstrating colocalization of FM 1-43FX (green) loading within focal punctate immunoreactivity of the glutamatergic synaptic vesicle marker (red), in wild type and mutant terminals, respectively. For illustrative purposes, further enlargements of selected puncta (white arrows) are included at far right. Note that in wild type and SNAP-25 deficient VGLUT1-positive terminals; detection of styryl dye uptake occurs easily, indicating that spontaneous AP-independent vesicular fusion remains intact in the absence of this t-SNARE. Similar results were found in *Snap25* null GABAergic terminals (E-F3), which maintained spontaneous vesicle recycling comparable to that of control neurons (G-H3)in the presence of TTX. I Quantitative analysis of the average FM 1-43FX fluorescence intensity per puncta of VGLUT1 and VGAT immunoreactivity during spontaneous AP-independent dye uptake in control (Con; wild type and heterozygous mice) and mutant (KO) terminals. Note that there is no significant difference between Con and KO neurons at terminals of either neurotransmitter phenotype. Data is a calculated average of the fluorescence intensity from 34 puncta used as a single value per animal. Following subtraction of background autofluorescence, a Wilcoxon signed rank test was used for statistical analysis. All values expressed as mean ± SD. (n=3 animals).

vesicular recycling at glutamatergic synapses of wild type neurons. Appreciable levels of styryl dye uptake, as marked by punctate FM1-43FX fluorescence, were also detected at VGLUT1-positive terminals of *Snap25*$^{-/-}$ neurons (Fig 5.5C-D3), indicating that, as in control cultures, glutamatergic synapses of these neurons also take up FM 1-43 in an AP-independent manner, even in the absence of SNAP-25-containing SNARE complexes. Importantly, robust punctate FM 1-43 fluorescence was also found colocalized with focal immunostaining for VGAT, demonstrating spontaneous vesicle recycling at GABAergic terminals of neurons in both control (Fig 5.5, panels E-F3) and *Snap25* knockout (panels G-H3) cultures. To quantify the relative extent of spontaneous vesicular recycling at glutamatergic and GABAergic terminals, I measured the intensity of FM dye fluorescence over individual immunoreactive puncta. Overall, I found that the levels of dye uptake was not significantly different between *Snap25*$^{-/-}$ and control neurons at either glutamatergic or GABAergic terminals (Fig. 5.5I). These data suggest, therefore, that spontaneous AP-independent vesicle fusion and endocytosis remains intact in both excitatory and inhibitory neurons lacking SNAP-25. Moreover, despite higher resting levels of calcium in the soma, styryl dye uptake in these terminals, and hence the size of the constitutive recycling vesicular pool of SNAP-25 deficient neurons, does not differ from wild type and heterozygous neurons.

Calcium-modulated, action potential-independent activity persists in *Snap25*$^{-/-}$ glutamatergic and GABAergic neurons

I found that after a 60 min incubation, *Snap25*$^{-/-}$ and control neurons exhibit similar levels of styryl dye reuptake corresponding to endocytosis resulting from spontaneous, action-potential independent vesicular fusion. This was surprising since it was shown previously that frequency of mPSCs, which should make up the bulk of the synaptic vesicle fusion events at synaptic terminals, is decreased in SNAP-25 deficient neurons (Washbourne et al., 2002; Tafoya et al., 2006; Bronk et al., 2007; Delgado-Martinez et al., 2007), as well as when SNAP-25 is cleaved with botulinum toxin (Capogna et al., 1997). However, mPSCs are indirect postsynaptic recordings of a presynaptic function, and the frequency of these events can be affected by several changes in synaptic development or physiology. Since in the absence of evoked stimuli and action potentials dye uptake would be expected to measure vesicles undergoing constitutive, AP-independent recycling, this suggests the relative size of this vesicular pool is not affected by the absence of SNAP-25 (Sara et al., 2005). Interestingly, previous studies have shown that the total number of vesicles is also not altered in either SNAP-25 null mutant neuronal synapses (Bronk et al., 2007; Delgado-Martinez et al., 2007) or chromaffin cells (Sorensen et al., 2003). Together, this suggests that SNAP-25 is not involved in the biogenesis of either vesicular pool responsible for evoked-stimulus driven or for ongoing AP-independent transmitter release. One possibility is that the decreased mPSCs frequency recorded from *Snap25*$^{-/-}$ mutant neurons may result from forming of fewer synaptic contacts,

since chronic blockade of AP-dependent transmission has been shown to decreased the number and maturation of functional synapses (Colin-Le Brun et al., 2004). Taken together with data from Chapter 3 (Tafoya et al., 2008), I conclude that while the ability to establish and maintain synchronous network activity is dependent on SNAP-25 expression, constitutive neuronal activity remains intact at the presynaptic terminal without expression of this SNARE protein.

6. References

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