

**DISSERTATION PROPOSAL**  
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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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**Attach 10 – 15 single-spaced, typewritten pages with the following information:**

1. **Background:** What are the most significant previous findings that impact on or motivate your research?
2. **Hypothesis**
3. **Specific Aims:** What are the specific aims of the dissertation research? Include the questions to be answered and the hypothesis to be tested.
4. **Methods and Experimental Design:** Outline your approach to the project, and describe the experimental techniques you will use.

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*Candidacy Proposal*

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

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## A. Specific Aims

The neural t-SNARE SNAP-25 is an important component of the SNARE core complex that mediates membrane fusion underlying neurotransmitter release. Previous results from mutant mouse models and pharmacological/neurotoxin blockades have demonstrated a critical role for SNAP-25-containing SNARE complexes in action potential (AP)-dependent release in several neurotransmitter systems, including glutamatergic, cholinergic, glycinergic, and catecholaminergic transmission, indicating that it is a crucial and possibly universal mechanism for neuroexocytosis. However, whether SNAP-25 is involved in evoked GABAergic transmission remains controversial. Based on findings in other neurotransmitter systems and consistent with its role in a central neuroexocytotic mechanism, I propose the hypothesis that action potential-dependent GABAergic neuroexocytosis requires SNAP-25-dependent facilitation of calcium-triggered transmitter release. To this end, I will examine the expression, function, and 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.** Previous electrophysiological data suggest that evoked GABAergic transmission was abolished in SNAP-25 knockout fetal brain. Because these findings could indirectly result from downstream effects on the development of GABAergic neurons, I propose to determine the expression of SNAP-25 in these cells by employing immunohistochemistry and fluorescent *in situ* hybridization (FISH) procedures. These experiments will examine whether SNAP-25 is comparably expressed in GABAergic and glutamatergic synapses. The results of these experiments have been reported (Tafoya et al., 2006).

**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<sup>-/-</sup> neurons.** Electrophysiological data show a lack of evoked transmission and decreased AP-independent spontaneous activity of GABAergic neurons in fetal SNAP-25 knockout brains. These recordings, however, depend on postsynaptic responses, which could be altered through compensatory mechanisms in the absence of evoked synaptic activity. Therefore, I will use western blotting and FM 1-43FX styryl dye uptake assays to determine whether these alterations in GABAergic transmission were due to a reduction in synaptic vesicle number/neurotransmitter content or because of the absence of a SNAP-25-dependent release mechanism. The majority of data from this specific aim has been published (Tafoya et al., 2006).

**Specific Aim 3: To determine the expression pattern of SNAP-25 isoforms in GABAergic neurons.** While the majority of neurons in the brain undergo a dramatic, developmentally regulated change in isoform expression, the relative levels of these isoforms can vary greatly amongst neurons within distinct adult neuroanatomical regions. Because this expression has not been characterized in any specific neurotransmitter system, analysis of isoform expression patterns in GABAergic neurons would be a unique investigation into the molecular machinery tailored to inhibitory neurotransmission. I propose to measure the relative levels of isoform expression in laser capture microdissected GABAergic neurons by the use of RT-PCR. Alternative methods to collect GABAergic neurons to assess SNAP-25 isoform mRNAs or proteins directly, using isoform specific antibodies are also proposed. If successful, this could lead to further studies beyond this proposal to characterize the shift in SNAP-25 isoforms in different brain regions during development.

## B. Background and Significance

**SNARE proteins in membrane fusion.** The soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptor (SNARE) complex plays a pivotal role in promoting neurotransmitter release through membrane fusion of synaptic vesicles. Although the precise mechanism through which the complex acts has yet to be fully resolved, it is well established that the neural SNARE complex responsible for the regulated exocytotic release of neurotransmitter is comprised of a four barrel coiled coil structure consisting of syntaxin 1a, SNAP-25, and vesicle associated membrane protein 2 (VAMP-2) (Sollner et al., 1993; Chen et al., 1999; Fukuda et al., 2000). Neural SNARE component proteins belong to a moderately sized protein family, whose members, defined for their association with either vesicle (v-SNARE) or target membrane (t-SNARE), mediate both constitutive and regulated exocytosis (For review, see Jahn et al., 2003). The neural SNARE complex, as a member of the SNARE protein family, is distinguished by conserved  $\alpha$ -helical domains that specifically direct the targeting of synaptic vesicles as well as facilitate membrane fusion for subsequent transmitter release during calcium-triggered neuroexocytosis (Weimbs et al., 1997; Bock et al., 2001; for review, see Sorensen, 2005). In addition to mediating a direct role in the fusion of transmitter-containing vesicles with the plasma membrane, this SNARE complex likely serves as a central scaffold that assembles accessory proteins to complete the fusion machinery required to orchestrate the highly regulated process of calcium-triggered neurosecretion (Melia et al., 2002; Nagy et al., 2005).

**The neural SNARE complex and its role in neurotransmitter release.** The critical role played by SNARE proteins, including SNAP-25, was originally identified by studies using tetanus and botulinum neurotoxins (TeNT and BoNT,

respectively; for review, see Montecucco et al., 2005). SNARE proteins are differentially cleaved in a site-specific manner by these natural toxins resulting in the abrogation of synaptic vesicular fusion, which initially defined the role of these proteins in neurotransmitter release. BoNT/A and /E subtypes specifically target SNAP-25 and disrupt the activity-dependent vesicular release of a wide variety of transmitters, including acetylcholine, glutamate, catecholamines and peptides, such as insulin and growth hormone (Graham et al., 2002; for review, see Montecucco et al., 2005). In addition, genetic ablation of the *Snap25* gene resulted in the abolishment of evoked glutamatergic and cholinergic transmission in neurons (Washbourne et al., 2002) and fast calcium-triggered catecholamine release from adrenal chromaffin cells (Sorensen et al., 2003) in homozygous mutant mice. However, despite a lack of evoked neuroexocytosis, *Snap25* null mutant neurons and chromaffin cells do retain stimulus-independent transmitter release (Washbourne et al., 2002; Sorensen et al., 2003). The persistence of SNAP-25 independent transmitter release suggests that alternative SNARE constituents, such as SNAP-23, can promote action potential (AP)-independent spontaneous vesicle fusion. It is interesting, however, that the frequency and amplitude of the AP-independent events recorded in mutant animals appear to vary amongst different neurotransmitter systems (Washbourne et al., 2002). Nevertheless, it is unclear whether these differences are due to alterations in the actual exocytotic events or by changes in the postsynaptic detection of transmitter (e.g. receptor sensitivity, abundance, or localization). Along with predominately excitatory neurotransmitters, glycine, an inhibitory messenger of the spinal cord, is not released in spinal cord cultures treated with BoNT/A (Keller et al., 2004), extending the evidence for SNAP-25-dependent calcium-triggered neuroexocytosis in several diverse neurotransmitter systems.

**Differential expression and function of SNAP-25 isoforms.** The t-SNARE SNAP-25 is a neuron specific protein that 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). The tandemly arranged exons, 5a and 5b, clearly arose by duplication early in vertebrate evolution and are highly homologous, differing by only nine amino acid residues in mammals, including humans (Bark et al., 1995). These amino acid differences include non-conservative changes within the N-terminal helical SNARE domain as well as the rearrangement of the four centrally located cysteine residues that serve as sites for palmitoylation and membrane association (Fasshauer et al., 1998). Because of the short intron length between exons 5a and 5b, the alternative splicing is obligatory and represents a binary molecular switch that determines the relative abundance of the two SNAP-25 isoforms in different brain regions, cell types, and time points during nervous system development. Moreover, inclusion of both exons into the mRNA would generate a codon frame shift and lead to a truncated protein with a different carboxyl terminus, which is not likely to be functional.

In the nervous system, SNAP-25a is the predominant form in early postnatal stages until a developmental change during the third week of life initiates abundant SNAP-25b expression that persists throughout maturation (Bark et al., 1995; Boschert et al., 1996; Jacobsson et al., 1999). In contrast, neuroendocrine cells, such as adrenal chromaffin cells do not undergo the dramatic developmental regulation and thus SNAP-25a remains the predominant isoform throughout development (Bark et al., 1995; Gonelle-Gispert et al., 1999; Grant et al., 1999). In addition to differences in development and amongst cell types, isoform expression occurs differentially amongst anatomical regions of the adult mouse brain, for example, as seen in distinct thalamic structures, a reciprocal pattern of expression occurs (Bark et al., 1995).

Differences in function between these two isoforms have been reported. For example, in mouse mutants which have persistent expression of SNAP-25a, short-term plasticity reflected by enhanced paired pulse facilitation is maintained similar to that found in a more juvenile brain (Bark et al., 2004). The rescue of SNAP-25 deficient chromaffin cells with SNAP-25b, compared to the earlier expressed SNAP-25a, leads to an increased size of the primed, readily releasable pool of vesicles (Sorensen et al., 2003). Structure function studies in chromaffin cells have shown that the functionally important residues reflect non-conservative, charge changes between SNAP-25a and SNAP-25b: H66Q (histidine to glutamine) and Q69K (glutamine to lysine) (Nagy et al., 2005). These critical residues are thought to face the outside of the 4-helix bundle of the SNARE complex, and it is likely that differences in function are mediated by differential recruitment of accessory proteins, rather than an intrinsic effect on the SNARE complex itself.

**SNAREs in GABAergic transmission.** Interestingly, it has recently been proposed by Matteoli and colleagues that GABAergic neurons employ a SNAP-25-independent mechanism of evoked vesicular fusion (Verderio et al., 2004; Frassoni et al., 2005). Initially, it was reported that SNAP-25 was not expressed in hippocampal GABAergic neurons and that vesicle recycling in these cells appeared to be resistant to BoNT/A treatment (Verderio et al., 2004). Subsequent results from this lab suggested that SNAP-25 immunoreactivity appears in the soma of cultured GABAergic neurons; however, this signal decreased and eventually disappeared over the course of 10 days of growth (Frassoni et al., 2005). In contrast, BoNT/E intoxication of isolated rat synaptosomes blocked stimulated GABA release during potassium-induced depolarization, indicating a failure of a SNAP-25-dependent mechanism (Ashton and Dolly, 2000).

With such variable results from toxin blockade assays, a targeted genetic mutation provides a reasonable alternative for determining SNAP-25's role in GABAergic transmission. In *Snap25* null mice, patch-clamp recordings of fetal (E17.5) mutants demonstrated that ablation of SNAP-25 eliminated evoked GABA<sub>A</sub> receptor-mediated postsynaptic responses while sparing the spontaneous AP-independent events, supporting SNAP-25's involvement in the Ca<sup>2+</sup>-triggered synaptic transmission of early developing GABAergic neurons (Tafoya et al., 2006). Further analysis addressing the requirement of SNAP-25 in evoked GABAergic transmission is detailed in the following aims.

### C. Progress Report:

**Specific Aim 1: To determine the expression and cellular localization of SNAP-25 in fetal and adult GABAergic neurons.** While previous electrophysiological studies by Mameli and Valenzuela have implicated that SNAP-25-containing SNARE complexes are required for GABAergic transmission in fetal brains, the localization of SNAP-25 to GABAergic presynaptic terminals still needs to be determined. 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. This aim attempts to demonstrate persistent SNAP-25 expression and its appropriate presynaptic localization in both fetal and adult GABAergic cells using immunostaining and fluorescent *in situ* hybridization (FISH). The results of this aim have been published (Tafoya et al., 2006).

**A. Is SNAP-25 persistently expressed in fetal GABAergic and glutamatergic neurons throughout 21 days *in vitro*?** To discern presynaptic terminals of neurons of different neurotransmitter phenotypes after fluorescent immunostaining, dissociated hippocampal neuronal cultures were employed. These cultures develop extensive processes and sufficiently fine networks of GABAergic and glutamatergic synapses needed to resolve well-defined immunoreactive punctate staining for analysis of SNAP-25 localization in these terminals.

#### **Experimental design and procedures:**

**Neuronal cell culture preparation.** Localization of SNAP-25 to presynaptic terminals of fetal GABAergic and glutamatergic neurons were examined by immunohistochemistry in dispersed neuronal cultures. The expression of SNAP-25 in GABAergic and glutamatergic synapses was established by dual immunostaining for specific markers that distinguish between the two neurotransmitter phenotypes. Dispersed neuronal cell cultures were prepared from E17.5 fetuses as previously described (Washbourne et al., 2002). Cultures were grown for 9-21 days *in vitro* (DIV) and then fixed in 4% paraformaldehyde (PFA).

**Glutamic acid decarboxylase (GAD) immunostaining.** GABAergic neurons were identified by immunostaining for the GABA synthetic enzyme, GAD. GAD is expressed as two isoforms, GAD65 and 67, which are encoded by separate genes and expressed in varying proportions by GABAergic neuronal subtypes. A rabbit polyclonal antibody raised against common sequences of GAD65/67 (Solimena et al., 1993) was a generous gift from Michele Solimena (Dresden University of Technology, Germany).

**Vesicular transporter staining.** Presynaptic GABAergic and glutamatergic terminals were distinguished using mouse monoclonal and rabbit polyclonal antibodies to vesicular GABA transporter (VGAT; Synaptic Systems, Göttingen, Germany) and the vesicular glutamatergic transporter 1 (VGLUT1; Synaptic Systems). Although 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) and while VGLUT1 is initially expressed at low levels, it is the predominant form in postnatal brain (Nakamura et al., 2005). VGLUT1 antibodies that do not cross-react with the two other isoforms were used, therefore, to track glutamatergic cells beyond 9–21 (DIV). To detect SNAP-25 expression, the SNAP-25 monoclonal antibody SMI 81 (Sternberger Monoclonals, Lutherville, MD) which shows specific, robust reactivity to a single 25 kDa protein band in wild type, but not SNAP-25 deficient neurons (See Tafoya et al., 2006; Fig. 1). Species appropriate Cy3-conjugated donkey anti-rabbit IgG and Alexa Fluor 488 donkey anti-mouse IgG antibodies were used for detection of primary antibodies.

**Measurement methods:** Images of immunofluorescent staining were obtained using a Bio-Rad 2100 Radiance confocal microscope with a 63X oil immersion differential interference contrast (DIC) objective (Numerical aperture; NA=1.4) at a resolution of 1024 X 1024 and an optical slice of 0.8 μm. Punctate staining in separate color channels was merged and quantitated for the degree of signal colocalization by using comparable threshold-level adjustments (MetaMorph 6.1 software; Universal Imaging, Downingtown, PA). Each value was determined using the average of three fields per animal (n=3 animals). Statistical analysis of colocalization was performed using one-way ANOVA with Bonferroni's post hoc comparisons (Prism 4 software; GraphPad Software, San Diego, CA). All values are expressed as mean ± SEM.

#### **Results and interpretation:**

*Analysis of colocalization.* In order to determine colocalization of immunostaining patterns, my 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. Because of differing sizes of punctate fluorescence between stains, not all pixels overlapped, resulting in percentages less than 100%. It is important to note the percentages, therefore, do not reflect the percentage of synapses that were double positive, but correspond to the total pixel overlap across each field.

*Coexpression of SNAP-25 in GABAergic and glutamatergic presynaptic terminals.* As shown in Figure 5A–B3 (Tafuya et al., 2006), confocal images of fluorescent immunostaining for SNAP-25 demonstrates that this t-SNARE is distributed throughout neuronal processes, but shows punctate staining consistent with its localization in presynaptic terminals. Immunostaining for GAD65/67 overlapped extensively with some of these SNAP-25-positive processes and presumptive terminals, suggesting their expression within the same synapse (Fig. 5A–B3, arrows and inset in merged image).

Neuronal cultures were stained with antibodies to the vesicular transporters VGAT and VGLUT1 to distinguish SNAP-25 expression in GABAergic and glutamatergic presynaptic terminals, respectively. As with GAD65/67, immunostaining for the two transporters VGAT and VGLUT1 resulted in a punctate pattern, consistent with synaptic localization of these vesicular proteins (Tafuya et al., 2006; Fig. 5C–F3). In addition, like GAD65/67 staining, the immunoreactive punctate staining for either transporter coincided with focal immunoreactivity for SNAP-25 (Fig. 5C–F3, arrows and digitally magnified inset), indicating that SNAP-25 is expressed in both terminals with GABA and glutamate-containing vesicles. As shown in Figure 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 DIV 21 of culture, indicating a persistent expression of this SNARE protein by GABAergic neurons.

*VGAT and VGLUT1 are not coexpressed in cultured fetal neurons.* Although 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). Because such a combined neurotransmitter phenotype could compromise the assignment of SNAP-25 to GABAergic and glutamatergic neurons, I examined whether cultured hippocampal neurons express both VGLUT1 and VGAT by dual immunostaining. 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. 5G,H; quantitated in I). This suggests that GABAergic and glutamatergic synapses were 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 were expressed in cultured hippocampal neurons and that SNAP-25 is present in the presynaptic terminals of both developing GABAergic and glutamatergic neurons.

**Limitations:** Although many studies have documented that dissociated cultures model the steps involved in synaptic maturation, they are limited to a few weeks of culture and may not reflect the same degree of synaptic and network connectivity that is established during development in an intact brain. Without investigation into GABAergic synapses of an adult brain, a transient expression of SNAP-25 solely during the initial stages of synaptic connectivity by developing inhibitory neurons in the absence of the appropriate neural environment remains a possibility. Therefore, I have addressed this limitation in the next set of experiments.

**B. Do GABAergic neurons in adult brain slices express SNAP-25 in presynaptic terminals?** The previous results demonstrate that SNAP-25 expression occurs in cultured fetal GABAergic neurons. To expand this analysis to mature GABAergic neurons, I used fluorescent immunohistochemistry to examine the expression and localization of SNAP-25 in GABAergic and glutamatergic neurons throughout cortical and subcortical regions of the adult brain.

**Experimental design:** Adult C57BL/6 mice [postnatal day >50 (>P50)] were anesthetized and fixed through transcardial perfusion of 4% PFA in accordance with standard methods. Thirty-micron coronal sections were prepared using a sliding microtome and immunostained using the same SNAP-25, VGAT, and VGLUT1 antibody combinations described in the previous section. Additionally, To-Pro-3 iodide was used as a nuclear counterstain to detect the soma of both neuronal and glial cells.

**Measurement methods:** Images were generated using the BioRad Radiance 2100 confocal microscope. Laser settings were optimized to acquire the linear range of fluorescence signal in our desired regions of interest, but due to the heterogeneity of neural tissue, this resulted in slight pixel saturation in neighboring regions that contained higher levels of

synaptic density. Regions with oversaturation of signal strength, however, were not used in the present analysis. Similar to my previous experiments, I used MetaMorph v6.1 software for quantification of the degree of pixel overlap and analyzed the data with Prism 4 statistical software using a one-way ANOVA with Bonferroni post hoc comparisons. All values expressed as mean  $\pm$  SEM (n=3 animals).

### **Results and interpretation:**

*SNAP-25, VGAT, and VGLUT1 staining in hippocampus.* As expected, in the hippocampus, there was a marked difference in the distribution of glutamatergic and GABAergic synapses. VGLUT1 immunoreactivity occurred primarily in the stratum oriens and stratum radiatum layers whereas VGAT staining primarily localized to the stratum pyramidale layer. Despite the predominant 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 each transporter with SNAP-25 immunoreactivity showed comparable colocalization between SNAP-25 and VGLUT1 (53%) or for SNAP-25 and VGAT (47%) (Tafuya et al., 2006; Fig. 6A–D, G). In contrast, little or no pixel colocalization was found after costaining for VGLUT1 and VGAT (Fig. 6E–G); consistent with separate and distinct GABAergic and glutamatergic synaptic terminals I previously observed in fetal hippocampal cultures.

*SNAP-25 expression in neurons of the thalamus and other regions.* As shown in Figure 7, A and B (Tafuya et al., 2006), immunofluorescent staining revealed a rich abundance of GABAergic synapses within the ventral posterolateral (VPL) nucleus of the thalamus. 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 expression of SNAP-25 within these interspersed glutamatergic terminals (Fig. 7C, D).

Overlapping punctate staining reflecting colocalized expression of VGAT/SNAP-25 was also observed throughout the cortex and caudate–putamen (data not shown). Again, as previously found for dispersed neuronal cultures, the punctate colocalized staining of VGAT and VGLUT1 with SNAP-25, coupled with the non-overlapping pattern and negligible signal colocalization of the two transporters, indicated that SNAP-25 is translocated to presynaptic terminals of both glutamatergic and GABAergic neurons in the adult CNS.

**Limitations:** Despite convincing overlap of SNAP-25 punctate staining with both VGAT and VGLUT1 immunoreactivity, it is difficult to assign SNAP-25 expression to specific GABAergic neuron populations based on synaptic localization. Because of the differential regulation of SNAP-25 expression amongst cell types and anatomical regions, it is therefore important to obtain a cell-to-cell comparison in order to determine coexpression in single GABAergic neurons. I determined this issue in the following experimental procedure.

**C. Are GAD65/67 and SNAP-25 mRNAs coexpressed in different GABAergic neuronal populations of the adult brain?** The previous immunofluorescent analysis demonstrated punctate, overlapping staining of SNAP-25 and VGAT/VGLUT1 in the neuropil, which presumably reflects synaptic terminals. However, these results could not be used to determine individual cellular expression of SNAP-25. Therefore, I performed FISH analysis of differently labeled SNAP-25 and GAD65/67 cDNA probes as an independent method to ascertain the expression of mRNA in the soma and thereby increase the cellular resolution necessary for cell-to-cell comparisons.

**Experimental design and procedures:** FISH was performed according to an established protocol (Guzowski et al., 1999; Vazdarjanova et al., 2002; Vazdarjanova and Guzowski, 2004). Briefly, 20-micron coronal sections of adult (>P50) mouse brain were obtained using cryostat sectioning. Sections were hybridized with digoxigenin-labeled SNAP-25 and fluorescein-labeled GAD65/67 antisense riboprobes, synthesized from transcription of cDNA plasmids in the presence of premixed RNA labeling nucleotides. After hybridization (overnight at 56°C), digoxigenin-labeled SNAP-25 riboprobe was detected using anti-digoxigenin HRP conjugate, amplified with TSA-biotin, followed by streptavidin-cyanine-3. The slides were treated with 2% H<sub>2</sub>O<sub>2</sub> to quench residual HRP activity and the fluorescein-labeled probe GAD65/67 was detected with an anti-fluorescein-HRP conjugate followed by and TSA-FITC amplification. Nuclei were counterstained with DAPI to identify the soma of GABAergic neurons.

**Measurement methods:** 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. 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. Statistical analysis was performed through

one-way ANOVA with Bonferroni's comparison using Prism 4 software. All values were expressed as mean percentage  $\pm$  SEM per region (n=3 animals).

**Results and interpretation:** As shown in Figure 8A (Tafuya et al., 2006), GAD65/67 was readily detected in neurons dispersed throughout layers I–V of cortex, consistent with the distribution of GABAergic neurons and the prominent hybridization for SNAP-25 mRNA within these cells. 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. 8B). 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. 8C).

In contrast, throughout neighboring regions, such as the 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. 8D). Quantitative analysis of the FISH images revealed that virtually all GAD65/67-positive neurons were also SNAP-25 positive throughout these four brain regions (Fig. 8E). Overall, these findings, in addition to results of the immunohistochemical analysis, indicate that mature GABAergic neurons maintain the expression of SNAP-25 throughout maturation.

**Limitations:** FISH analysis provides further evidence of SNAP-25 mRNA expression in adult GABAergic neurons, consistent with immunological detection of proteins in neuropil and presumably nerve terminals. It, however, does not address a functional role of this t-SNARE protein in neuroexocytosis within the adult animal. Despite normal brain development throughout gestation, SNAP-25 deficient mutant mice die at birth, making it impossible for functional analysis at a more mature developmental time point. The development of a conditional SNAP-25 knockout (now in progress) or an inducible RNAi knockdown system in viable mice would be needed to address this question adequately. Nevertheless, the collective findings from previous studies showing that SNAP-25 deficient neurons lack evoked GABAergic synaptic transmission, along with the results shown here that SNAP-25 protein and mRNA colocalizes equivalently with glutamatergic and GABAergic synapses and soma in not only fetal, but also adult neurons, provide strong evidence that SNAP-25 is critical for transmitter release in GABAergic inhibitory and glutamatergic excitatory neurons.

**Specific Aim 2: To determine whether stimulus-evoked, but not action potential-independent, vesicular recycling is blocked equally in both glutamatergic and GABAergic synapses of Snap25<sup>-/-</sup> neurons.** This aim will address the role of this neural t-SNARE protein in neuroexocytosis of GABAergic vesicles. Previous electrophysiological studies demonstrated a lack of evoked GABAergic transmission in SNAP-25 deficient fetal brains as well as a decrease in both frequency and amplitude of spontaneous action potential-independent mini's, and interestingly, increased response to exogenously applied GABA. However, because these recordings depend on postsynaptic responses, which could be altered through compensatory mechanisms in the absence of evoked synaptic activity, I examined, using western blot analysis, whether the lack of AP-dependent transmission results from a decrease in the number synaptic vesicles within SNAP-25 deficient GABAergic neurons. Furthermore, to determine the functional role of SNAP-25 in GABAergic vesicular recycling, I used an FM 1-43FX styryl dye uptake assay to examine whether stimulus evoked, but not AP-independent, coupled exo/endocytosis was equally blocked in both VGAT- and VGLUT1-immunopositive synapses. The results of the experiments described in sub aims 2a and 2b have been published (Tafuya et al., 2006).

**A. Is the relative amount of GABAergic and glutamatergic vesicles altered in Snap25 null neurons.** A lack of evoked GABAergic transmission despite the presence of highly responsive GABA<sub>A</sub> receptors (Tafuya et al., 2006) could be due to specific deficits in synaptic vesicles of SNAP-25 deficient GABAergic neurons. To determine whether alterations in evoked GABAergic transmission were due to decreased numbers of GABAergic vesicles, I performed a series of western blots to compare the expression of VGAT, VGLUT1, and synaptophysin, a general marker for synaptic vesicles, in mutant and control fetal brain.

**Experimental design:** Crude synaptosomal fractions (LP2) of hippocampus and cortex were prepared from E17.5 mutant and control fetuses by standard methods (Huttner et al., 1983) and fractionated by SDS-PAGE gel electrophoresis followed by western blotting. Statistical analysis was performed through one-way ANOVA with Bonferroni's comparison using Prism 4 software. All values were expressed as mean percentage  $\pm$  SEM per region (n=3 animals).

**Results and interpretation:** As shown in Figure 3 (Tafuya et al., 2006), mutant mice expressed VGLUT1, VGAT, and synaptophysin at comparable levels to control animals. Quantifying the signal intensity for the vesicular transporter relative to the intensity of synaptophysin, moreover, showed no difference between mutant and controls, and further, that

these SNAP-25 deficient animals do not have a specific reduction in the relative amount of GABAergic, as well as glutamatergic synaptic vesicles.

**Limitations:** While this analysis can provide a measure of relative vesicle number in GABAergic neurons, it cannot describe the state of neurotransmitter content in these vesicles. The decrease of amplitude and frequency of GABA mPSCs in SNAP-25 mutant brain could be compromised through disturbances in transmitter filling, such as alterations in transporter function, which is driven by a proton gradient created by vesicular proton pumps. In addition, decreases in glutamine, the substrate for GABA synthesis could occur as a systemic effect in the absence of evoked glutamatergic neurotransmission (Mathews and Diamond, 2003). These possibilities could be evaluated through a series of additional experiments that directly measure GABA content of vesicles. Additionally, a more direct approach for measuring synaptic vesicle number would be by electron microscopy, which is able to resolve the vesicle number within individual synapses. Although mature inhibitory and excitatory synapses are easily identified by their morphology (symmetrical synapses and asymmetrical synapses, respectively), SNAP-25 knockout mutants die at birth, making this type of analysis on mature GABAergic neurons impossible. Such investigations, however, lie outside the interests of the present proposal and are not required to resolve its central hypothesis.

**B. Does the lack of SNAP-25 abrogate action potential-dependent vesicular recycling in GABAergic and glutamatergic presynaptic terminals?** *Snap25* null fetal brains do not display GABAergic postsynaptic responses following field stimulation, despite the presence of functional GABA<sub>A</sub> receptors (Mameli and Valenzuela in Tafuya et al., 2006). This could result from stimulus-induced exocytosis of empty vesicles rather than a lack of the necessary exocytosis machinery itself. I, therefore, examined whether activity-dependent exocytosis in both GABAergic and glutamatergic synapses was blocked equally in neurons lacking SNAP-25 by measuring FM 1-43 dye uptake. As opposed to electrophysiology techniques that use a postsynaptic neuron as a sensor of presynaptic activity, neuroexocytosis can be measured directly in the presynaptic cell as styryl dye is loaded after membrane fusion into recycling synaptic vesicles.

**Experimental Design:** Hippocampal cultures were prepared from *Snap25*-null mutant, heterozygote, and wild type E17.5 fetuses as described in Specific Aim 1. Homozygous mutants were initially identified by morphology and lack of hind limb pinch response, with subsequent genotype determination by PCR (Washbourne et al., 2002). In order to determine styryl dye uptake within GABAergic and glutamatergic terminals, I used the aldehyde fixable analog FM1-43FX that is compatible with subsequent fluorescent immunohistochemistry with VGAT and VGLUT1 antibodies. The use of amphipathic styryl dyes has been reviewed extensively (Brumback et al., 2004) and exploits the increase in fluorescence when the dye is associated with the hydrophobic environment of cell membranes. Although the dye is membrane impermeable, it is readily taken up through endocytosis, whereas dye bound to external plasma membrane is effectively removed by washing. In addition, specificity of uptake by synaptic vesicles can be determined through a subsequent round of depolarization in the absence of FM 1-43, which allows synapses to jettison the encapsulated dye during vesicular recycling. In the presence of receptor antagonists APV, CNQX, and bicuculline, neurons were loaded with FM 1-43FX dye by either high potassium depolarization (90 mM) for 90 sec (Sara et al., 2002) or hyperosmotic shock by sucrose application (500 mOsm) for 30 sec (Rosenmund and Stevens, 1996). Cells were washed and either destained by undergoing a second round of high potassium depolarization (2 min) in the absence of FM 1-43FX or fixed immediately in 4% PFA and immunostained. Statistical analysis was performed through one-way ANOVA with Bonferroni's comparison using Prism 4 software. All values were expressed as mean percentage  $\pm$  SEM per region (n=5 animals).

**Measurement methods:** Coverslips were visualized with a Zeiss LSM 510 META/Axiovert 100M laser confocal microscope using a 63X oil DIC objective (NA, 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. FM1-43FX fluorescence intensity that colocalized within the immunoreactive punctate staining of either VGAT or VGLUT1 was measured. After subtraction of background fluorescence, data were analyzed using one-way ANOVA with Bonferroni's post hoc comparisons. All values are expressed as mean  $\pm$  SEM.

**Results and Interpretation:** As shown in Figure 4 (Tafuya et al., 2006), wild type cultures showed robust punctate FM dye fluorescent staining after depolarization that colocalized with the punctate staining pattern for VGLUT1 (A, B1–B3) and VGAT (D, F1–F3). In contrast, no FM dye uptake was detectable in SNAP-25 knockout neurons at either glutamatergic or GABAergic immunolabeled synapses (Fig. 4C, G; D1–D3, H1–H3, respectively). Similar images were obtained for wild type and mutant neurons after application of hypertonic sucrose (data not shown).

Quantitation of the fluorescence intensity of FM1-43 dye that colocalized to VGLUT1- and VGAT-positive terminals in response to either depolarization (Fig. 4I) or hyperosmotic shock (4J) confirmed that both glutamatergic and GABAergic synapses in wild type neurons readily endocytosed FM1-43 dye, which could be effectively unloaded after a second round

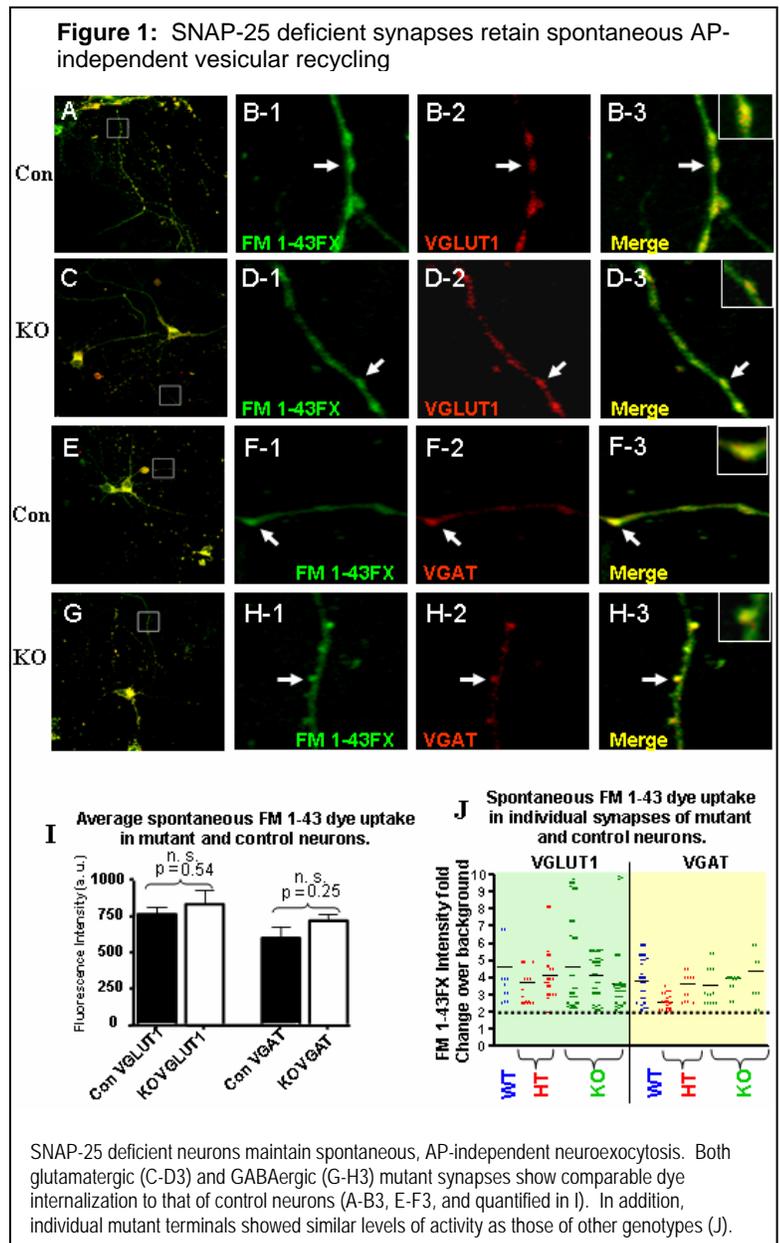
of depolarization-triggered exocytosis (e.g., destain in I and J). Importantly, neither VGLUT1- nor VGAT-containing synapses of SNAP-25-deficient neurons showed significant uptake above background fluorescence after application of high  $K^+$  or sucrose, demonstrating a lack of stimulus driven endocytosis and therefore highly compromised neuroexocytosis in both GABAergic and glutamatergic synapses. Together, these results suggest that the lack of evoked PSCs seen in electrophysiology recordings reflects a defect in vesicular fusion in *Snap25*<sup>-/-</sup> neurons, and is not attributable to an alternative mechanism, such as the recycling of transmitter-depleted synaptic vesicles.

**C. Is SNAP-25 required for AP-independent vesicular recycling at GABAergic and glutamatergic synapses?** The previous experiment confirmed that a lack of SNAP-25 results in a loss of stimulus driven vesicular fusion, and hence, AP-dependent neurotransmission was blocked, resulting in abolishment of evoked PSCs during field stimulation. However, the decrease in amplitude and frequency of GABAergic minis in mutant fetal brains may be the result of an indirect effect that is disturbing the neurotransmitter content of vesicles rather than synaptic vesicle fusion per se. The question remains whether AP-independent neuroexocytosis in GABAergic neurons exist and whether it is comparable to those of glutamatergic cells. Therefore, I examined AP-independent neuroexocytosis by measuring spontaneous FM1-43 uptake in the presence of tetrodotoxin (TTX), which blocks voltage-gated sodium channels, and consequently, action potential propagation.

**Experimental design:** This experiment was designed similar to the previous assay as DIV10-12 *Snap25*<sup>-/-</sup> and control E17.5 hippocampal cultures were loaded with FM 1-43FX dye. However, the dye was loaded through AP-independent spontaneous activity at 37°C for 60 min. with CNQX, APV, bicuculline, and TTX to block action potential propagation. Cultures were then fixed, immunostained, and imaged as above.

**Results and interpretations:** Control neuronal cultures (See Fig. 1A-B3, E-F3 of this proposal) were imaged with fluorescent confocal microscopy as described in the previous sub aim. FM 1-43 FX (green) and VGLUT1/VGAT (red) are shown individually and merged (far right) demonstrating FM 1-43FX uptake in both glutamatergic and GABAergic synapses. As in control cultures, mutant neurons (Fig 1C-D3, G-H3) showed appreciable focal accumulation of FM dye that colocalized with both VGLUT1 and VGAT signal. When quantified, dye uptake in both glutamatergic and GABAergic mutant synapses was similar compared to control neurons (Fig. 1I). Thus, as seen in several other neurotransmitter systems, including glutamatergic transmission, there is a low level of SNAP-25 independent vesicular turnover, which is likely to contribute to AP-independent spontaneous synaptic transmission at GABAergic terminals.

To quantify activity at individual synapses, I measured FM dye fluorescence per immunoreactive puncta, and using a ratio of punctate FM dye fluorescence over the background staining of neurites, I arbitrarily defined synapses as being active if their FM fluorescence was more than two-fold greater than background. Any synapses that fell below this criterion were considered dormant. As shown in Figure 1J, individual SNAP-25 deficient GABAergic terminals maintained spontaneous vesicular recycling and internalization of FM 1-43 at levels comparable to both wild type and heterozygous neurons. This indicates that the decrease in mini frequency and amplitude observed in *Snap25*<sup>-/-</sup> mutant brain slices is not due to a lack of SNAP-25-independent spontaneous vesicular fusion,

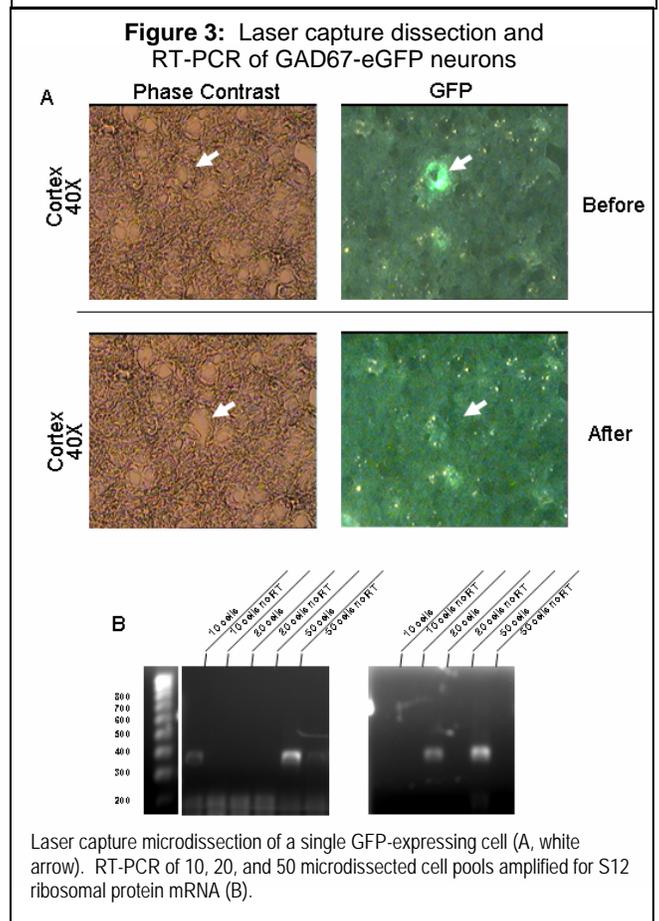
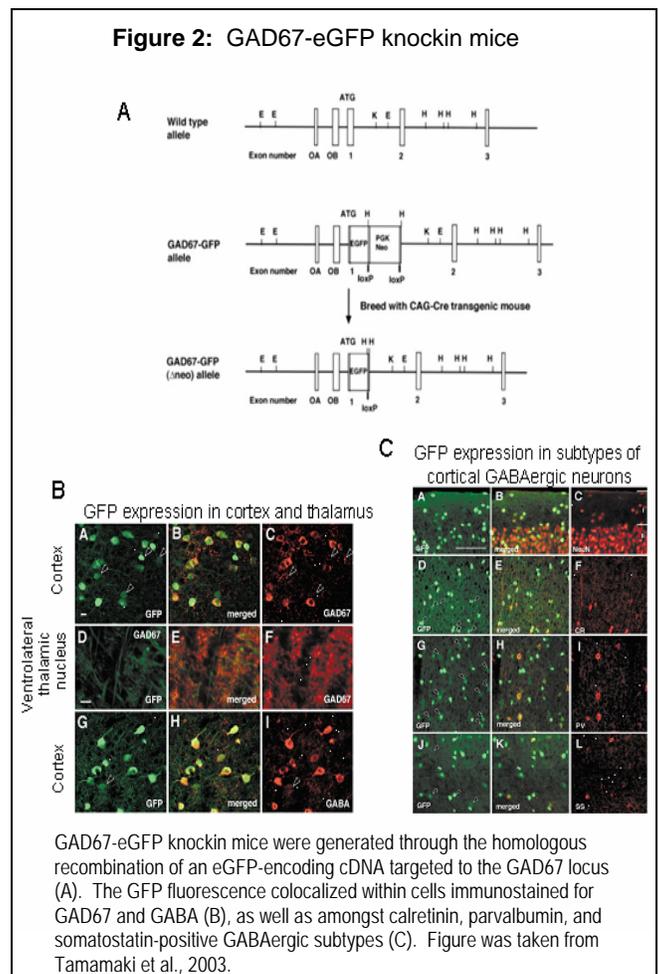


but likely represents a deficiency in the GABA content of synaptic vesicles.

**Specific Aim 3: To determine the expression pattern of SNAP-25 isoforms in GABAergic neurons.** Although SNAP-25 isoforms are expressed in a tightly regulated manner during brain development, certain regions of adult brain continue to express SNAP-25a into adulthood (Bark et al., 1995), suggesting that the isoforms could contribute to the diversity of mature neurotransmission. As mentioned previously, functional differences have been discovered between SNAP-25a and SNAP-25b that may underlie the specific activity profile of certain neurons. GABAergic neurons have distinctive activity patterns and abilities in order 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, and a higher calcium influx upon depolarization (Verderio et al., 2004) and a slightly depolarized resting membrane potential for fast activation upon excitation compared to other neurons (reviewed by Jonas et al., 2004). In addition, recent evidence suggests that the presynaptic proteins Munc13-1, RIM1 $\alpha$  (Rab3-interacting molecule 1 $\alpha$ ), and synapsin exert different modulatory effects on glutamate and GABA neurotransmission that shape the physiological parameters governing synaptic activity (Augustin et al., 1999; Schoch et al., 2002; Gitler et al., 2004), indicating that a specific constellation of protein effectors is used during GABA release. Thus, mature GABAergic neurons may continue to express the functionally distinct SNAP-25a as the predominant isoform in order to mediate their specific activity demands.

Isoform expression has thus far not been examined within the context of neurotransmitter phenotype, and so, aside from a thorough classification of SNAP-25 expression, the analysis of isoform expression patterns in GABAergic neurons could provide further insight into the specification of molecular machinery tailored to inhibitory neurotransmission. I propose to measure the relative levels of isoform expression by RT-PCR in laser capture microdissected GABAergic neurons to examine this issue. I will focus on four anatomical regions, the cortex, hippocampus, caudate, and thalamus, in order to analyze the major GABAergic populations within the brain. This approach, using well-defined regions and neural circuits, will provide insight into regional differences in SNAP-25 isoform expression.

**Experimental design:** For this experiment, I plan to use adult (PN>50) GAD67-eGFP knockin mice provided by Dr. Nobuaki Tamamaki (see Fig. 2A of this proposal). These mutants are reported to exhibit GFP fluorescence in all calretinin-, parvalbumin-, and somatostatin-positive GABAergic cell populations throughout layers I-V of the cortex, as well as throughout hippocampus, substantia nigra, thalamus, cerebellum, olfactory bulb, caudate putamen, and globus pallidus (Tamamaki et al., 2003). The GFP fluorescence closely follows GAD67 *in situ* hybridization patterns as well as immunohistochemistry staining for inhibitory markers throughout development (Figure 2B-C). As shown in Figure 3A (of this proposal), these mutant mice allow the specific targeting



and expression analysis of GABAergic neurons by laser capture microdissection and RT-PCR analysis. In addition, using the morphology of pyramidal cells, GFP-negative glutamatergic neurons will be collected in order to compare SNAP-25 isoform expression levels.

Animals will be transcardially perfused with 4% PFA followed by dissection and overnight post-fixation of the brain. Eight-micron thick coronal sections will be acquired using a cryostat followed by the collection of GFP-positive GABAergic neurons by laser capture microdissection. RT-PCR analysis will be carried out on captured cells using several primer sets: S12 ribosomal protein (loading control), GFP (GAD67 specific), VGAT (GABAergic neuron specific), VGLUT1 (glutamatergic neuron specific), SNAP-25, SNAP-25a, SNAP-25b, and GFAP (glial cell specific). These primer sets will allow for multiplexing, if needed, and will determine possible glutamatergic neuron and glial cell contamination when assessing relative levels of SNAP-25 isoform expression in GABAergic neurons. RNA from collected cells will be extracted using a commercial kit (PicoPure RNA isolation kit; Molecular Devices Corporation, Sunnyvale, CA). cDNA will be synthesized from RNA extracts using Superscript First-Strand RT kit (Qiagen, Valencia, CA) and amplified for 40 cycles. I plan to use three animals with 50 GFP-positive cells collected from each of the four brain regions mentioned previously.

Preliminary studies have demonstrated the feasibility of microdissecting single GFP-fluorescent GABAergic neurons and RT-PCR amplification of specific RNA transcription from these captured cells (Fig. 3B of this proposal).

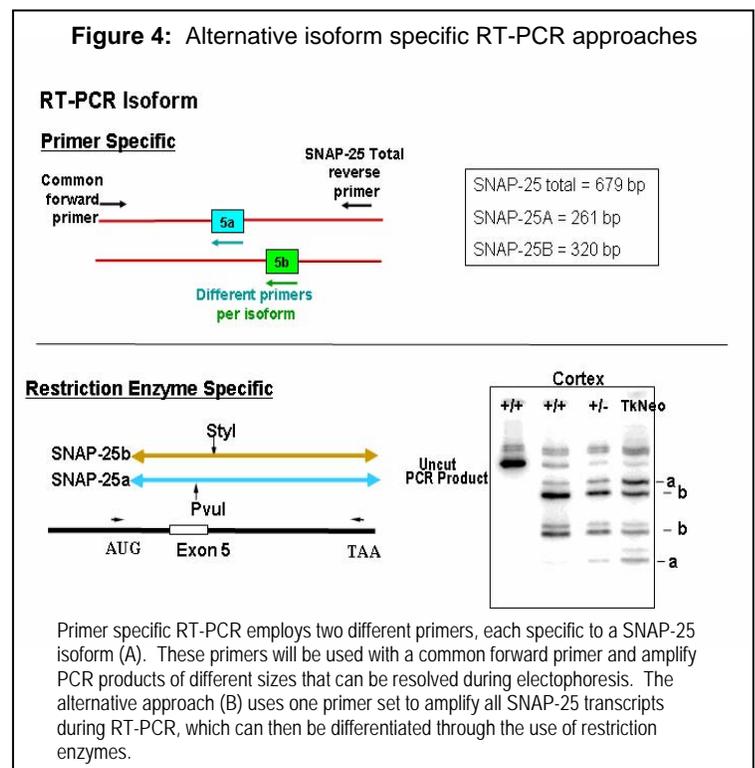
**Limitations and Alternative approaches:** This method of RT-PCR will rely on the ability of isoform specific primer sets to provide detectable levels of amplification (Fig. 4, upper panel, of this proposal). If problems in the specificity of amplification using these primer sets should arise, I will use an established RT-PCR protocol that makes use of different restriction sites encoded within the sequence of the two versions of exon 5 (Bark et al., 2005; depicted in Fig. 4, lower panel, of this proposal). The advantage of this method is that it uses only one primer specific to SNAP-25, thus avoiding possible primer competition for nearly identical transcripts while allowing for the measure of the relative differential expression of isoforms by quantifying radiolabeled bands specific to each isoform after electrophoresis.

In addition, two alternative approaches to laser capture microdissection are FACS sorting of GFP-expressing GABAergic neurons from dissociated neural tissue (Tomomura et al., 2001) and RNA harvesting of single cells by patch pipette (reviewed by Sucher et al., 2000). FACS cell sorting is likely a more viable alternative as it would quickly isolate many GABAergic neurons as

compared to the time intensive process harvesting RNA from individual cells through patch pipetting. The disadvantage of FACS cell sorting is that GABAergic neurons from different areas of the brain would be grouped together, decreasing the ability to detect regional differences in expression. In addition, harsh dissociation procedures used when creating a single cell suspension could destroy cells, allowing mRNA contamination from other neurons to alter the results.

**Expectations and possible extensions:** I expect mature GABAergic neurons to express SNAP-25b predominantly, as seen globally in the adult brain. However, if it were determined that SNAP-25a is the major isoform in GABAergic neurons, this specific aim would provide a novel insight into the molecular machinery required for inhibitory neurotransmission and provide the initial groundwork for future investigations outlined below.

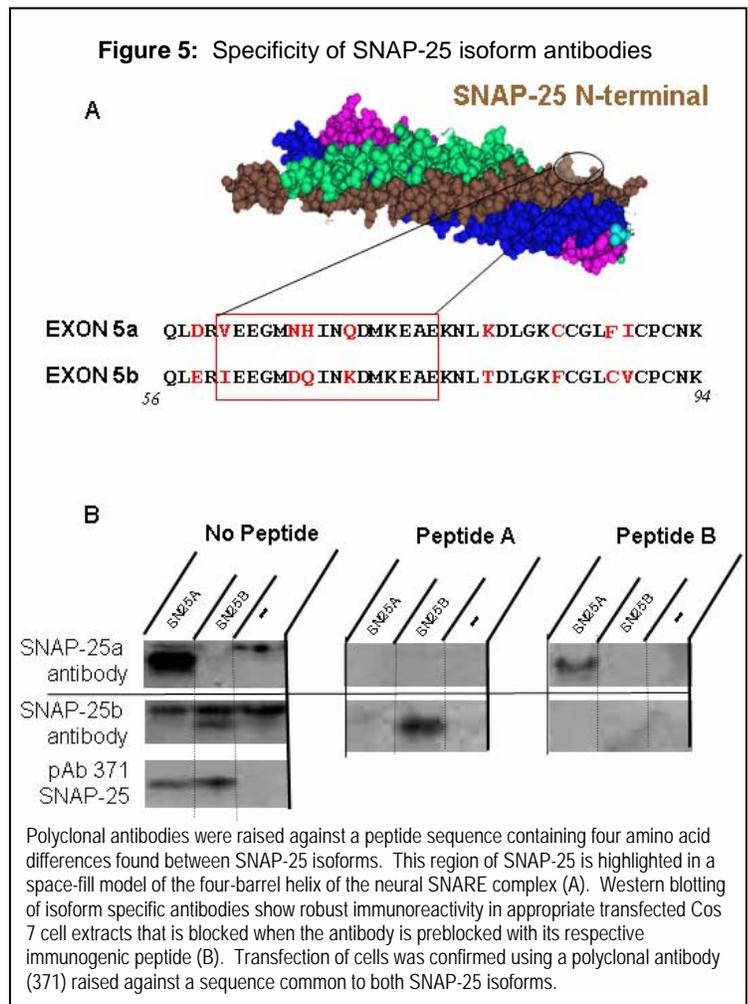
**Immunohistochemistry with isoform specific antibodies.** I have developed a novel set of rabbit polyclonal antibodies that can distinguish between SNAP-25a and SNAP-25b protein. These antibodies were raised against synthetic peptides containing sequences (residues 60-75) that differ in four amino acids. 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 complex, potentially enabling us to detect the isoforms when inserted in an assembled SNARE complex (Fig. 5A of this proposal).

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. 5B of this proposal). 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 not been successful. 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: Specificity of SNAP-25 isoform antibodies**



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