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UNIVERSITY OF CALIFORNIA, SAN DIEGO

Immunohistological Study of Sorting Nexin 27 Expression in Rat Brain

A Thesis submitted in partial satisfaction of the
Requirements for the degree Master of Science

in

Biology

by

Kalyn Michiko Stern

Committee in charge

Professor Paul Slesinger, Chair
Professor Gentry Patrick, Co-chair
Professor Yimin Zou

2008

The Thesis of Kalyn Michiko Stern is approved and it is acceptable in quality and form for publication on microfilm:

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Co-chair

_____

Chair

University of California, San Diego

2008

DEDICATION

I dedicate this work to all those people in my life who have managed to keep me alive and sane all these years.

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LIST OF ABBREVIATIONS

ABC	Avidin Biotin Peroxidase Complex
CA 1-4	Cornu ammonis 1-4
DAB	3’3 diaminobenzide
DG	Dentate gyrus
GFAP	Glial fibrillar associated protein
GIRK	G-protein gated inwardly rectifying potassium channels
HEK 293	Human embryonic kidney cells
LTD	Long term depression
LTP	Long term potentiation
MAP	Methamphetamine
MAP-2	Microtubule associated protein-2
mRNA	Messenger RNA
Mrt1	Methamphetamine responsive transcript 1
NeuN	Neuronal nuclei marker
NRS	Normal rabbit serum
NT	No treatment/ untransfected
PSD	Post synaptic density
PtdIns	Phosphoinositides
PX	Phox
sIPSP	Slow inhibitory postsynaptic potential
SNX	Sorting nexin

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ABSTRACT OF THE THESIS

Immunohistological Study of Sorting Nexin 27 Expression in Rat Brain

by

Kalyn Michiko Stern

Masters of Science in Biology

University of California, San Diego, 2007

Professor Paul Slesinger, Chair
Professor Gentry Patrick, Co-chair

Drug sensitization is thought to arise through changes in transcription, modification of signaling, and synaptic transmission. A novel rat gene, *mrt1,* may contribute to this neuronal plasticity as it is upregulated during sensitization. Mrt1 contains a PX domain which classifies it as a sorting nexins- thus mrt1 is also called SNX27. In addition, SNX27 also has a PDZ domain which has been demonstrated to interact with and mediate GIRK channel trafficking. To explore this interaction and the role that SNX27 may play in sensitization it is necessary to localize SNX27 expression. Using a novel SNX27 antibody I had three aims for my Masters project. First, to characterize the specificity of the antibody. Second, to characterize SNX27 expression

in the brain, particularly in the hippocampus. Third, to correlate this expression back to an interaction with GIRK channels. The SNX27 antibody demonstrated good specificity for SNX27 protein. SNX27 was found to be ubiquitously expressed in nearly all regions of the brain, with particularly noticeable expression in the hippocampus. SNX27 was expressed in the principle cells of the DG, CA3, and CA1; as well as in glia throughout the hippocampus. Colocalization studies suggested possible SNX27 expression in cell bodies, dendrites, and axons. Perisomatic punctate expression was also seen, suggesting a vesicular localization of SNX27. GIRK3 expression was mostly axonal and did not correlate well with SNX27 expression. However, punctate GIRK3 expression was seen in cells displaying similar SNX27 expression, alluding to possible overlap in GIRK3 and SNX27 vesicular expression.

CHAPTER 1: Introduction

1.1 Drug Addiction

Modern molecular techniques have allowed greater insight into the molecular and cellular underpinnings that contribute to drug addiction. Recent advances in the field have demonstrated that addiction is the result of a complex interaction of several components including both the primary targets of drug action and genes that regulate events further removed from the initial sites of action. Acting through nearly all the components of the brain's reward pathway, psychoactive drugs, such as amphetamines, elicit a wide spectrum of altered behaviors including; decreased fatigue, elevated mood, and induction of a euphoric state (Gilman et al., 1980). Like humans, the reward pathways of animals are similarly affected by drug usage. Thus, rodents, insects, and fish provide good models for the study of drug addiction (Mohn et al., 2004). Such studies have led to the recognition of several phases and aspects of addiction including; the acute drug response, sensitization, place preference, tolerance, withdrawal, self-administration, extinction, and relapse (Mohn et al., 2004).

Sensitization

In rodent models of addiction, administration of amphetamine results in hyperlocomotion and stereotypy through the activation of the reward pathway (Mohn et al., 2004). The reward pathway includes the nucleus accumbens, striatum, ventral tegmental area (VTA), prefrontal cortex and amygdale (Kaji et al., 2003) (figure 1).

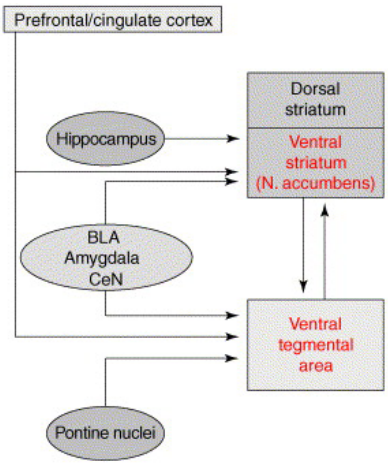

Figure 1. Schematic of brain reward/addiction pathway. Traditionally implicated components of the reward pathway (red) include the VTA and Nucleus Accumens. However other regions in the midbrain have been recognized to interact in this pathway. Image adapted from Jones and Bonci (2005).

Depending on the pattern of drug intake, hyperlocomotion and stereotypy can either decrease (tolerance) or increase (sensitization) with subsequent usage. If the drug is administered intermittently, rather than continuously, sensitization is more likely to result (Robinson and Becker, 1986). The enhanced behavioral response that occurs in sensitization is likely due to neuronal plasticity- caused by lasting changes in transcription, modification of signaling, and synaptic transmission (Mohn et al., 2004). Evidence suggests that such changes may occur in regions of the brain correlated with associative learning and typical memory formation (Jones and Bonci, 2005). Thus addiction may alter synaptic connectivity in a fashion similar to that recognized in memory formation. Studies have implicated a wide variety of genes that may be responsible for such modification. For example, the scaffold protein PSD-95, which is abundant in glutamatergic synapses, was found to have altered expression in both pharmacologically and genetically sensitized animals (Mohn et al., 2004). As further

studies are conducted the list of components involved in sensitization continually grows.

Methamphetamine Responsive Transcript (mrt1)

A particular study aimed at identifying possible genes involved in sensitization identified a novel rat gene- *mrt1* (methamphetamine responsive transcript 1). Discovered from samples of rat neocortex using RNA arbitrarily primed PCR (RAP-PCR), *mrt1* was found to be both developmentally regulated and methampthetamine (MAP) sensitive. *mrt1* has two splice variants: alpha-isoform (*mrt1a*) and beta-isoform (*mrt1b*), which vary only slightly in their c-terminal tail. Both variants display developmental regulation, with peak expression in early development and levels significantly decreasing thereafter. Acute MAP injection leads to a significant increase *mrt1b* mRNA in a time dependent induction manner, with peak induction occurring 1hr post-injection, with no significant increase detected following that hour. This MAP dependent upregulation also has a developmental component as well, as induction only occurs in adult rats. In contrast, *mrt1a* mRNA shows no induction upon acute MAP injection. Subcellular localization of Mrt1 protein suggests a synaptosomal distribution of Mrt1b; whereas, Mrt1a does not display regional specificity for either the synapse or the postsynaptic density. This difference in expression pattern is mimicked in regional northern blots with *mrt1b* mRNA expressed in various brain regions and *mrt1a* mRNA found primarily in the testes (Kaji et al., 2003).

Mrt1 contains an RA, PDZ, and PX domain (Kaji et al., 2003). The RA (**R**as-**a**ssociated) domain is a GTP to GDP exchange factor that may act on small Ras-like G proteins. The other two domains, the PX and PDZ, are a unique combination of domains found in no other known protein (Kaji et al., 2003). PDZ domains are protein-protein interacting domains which often act as scaffold proteins (Kim and Sheng, 2004). PX domains are lipid-lipid interacting domains (Seet and Hong, 2006). The PX domain is a defining attribute which classifies PX-containing proteins into a large family of proteins called sorting nexins (SNX) (Carlton et al., 2005); thus, mrt1 is also known as SNX27 (figure 2).

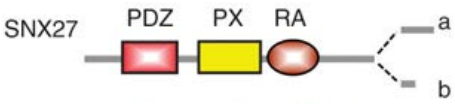

Figure 2. Schematic of SNX27. SNX27 has three domains; a PDZ, PX and RA domain. There are two splice variants, **a** and **b**. Iso-form **a** has a slightly longer c-terminal tail.

1.2 Sorting Nexins (SNX)

Sorting nexins are an emerging and growing field of proteins classified not by function but by the presence of a Phox-homology (PX) domain. SNXs were first identified in 1996 when Gordon Gill isolated sorting nexin-1 (SNX1), the mammalian homology of the yeast vacuole sorting protein Vps5p. Vps5p is part of the yeast retromer complex, which along with several other proteins, Vps17p, Vps26p, Vps29p, andVsp35p, functions to regulate endosome to Golgi retrieval. SNXs are similarly implicated in cellular trafficking. However, the SNX family did not really being to grow until the discovery of the PX domain in 2001 (Carlton et al, 2005).

PX Domains

The PX domain was named after, and first identified as, a conserved motif of approximately 130 amino acids present in the $p40^{phox}$ and $p47^{phox}$ subunits of the **ph**agocyte NADPH **ox**idase (Ponting, 1996). The structural PX domain interacts with phosphoinositides (PtdIns) and is thus a lipid-lipid binding domain (Song et al., 2001). PX domains are often involved in trafficking between the various membranous bound compartments within the cell which contain enriched levels of PtdIns. The family of SNXs are an interesting group of PX containing proteins as they preferentially associate with PtdIns(3)P (PI3P) (Boeddinghaus et al., 2002). PI3P is a phosphoinositide enriched on the cytosolic face of early endosomes (Gillooly et al.,

2000) giving this lipid a significant role in the sorting processes of endocytic and biosynthetic pathways (Seet and Hong, 2006).

The SNX family is comprised of 30 proteins (Teasdale et al., 2001) which can be further broken down into 3 subgroups. The first group consists of SNXs with a PX domain with minimal flanking regions (SNX3, SNX10, SNX12, SNX22 and SNX24). The second group consists of proteins containing the PX domain and a C-terminal coiled coil region that is related to the BAR (Bin/Amphinphysin/Rvs) domain (SNX1, SNX2, SNX4, SNX5, SNX6, SNX7, SNX8, SNX9, SNX18, SNX30, SNX32, and SNX33). The third group consists of proteins in which the PX domain is flanked by other varying structural motifs. This last group of SNXs consists of 13 proteins (SNX11, SNX12, SNX14, SNX15, SNX16, SNX17, SNX19, SNX20, SNX21, SNX25, SNX27, SNX29, and SNX31). Due to the wide variety of region composition in the third group the cellular function of many of these SNXs remains to be studied. Amongst these, SNX27 is an emerging protein of interest as it is the only SNX to contain a PDZ domain (Seet et al., 2006).

PDZ Domain

The PDZ domain was discovered as a sequence of repeats in the primary structures of the post-synaptic density 95 (**P**SD95), disk-large (**D**lg) and zona occludens-1 (**Z**O-1) proteins (Cho et al., 1992). Since then, this approximately 90 amino acid long domain has been identified in many other proteins with estimates of more than 400 different PDZ domains existing in humans and mice. Often occurring

in multiple copies within a protein, these tandem arrays of linked PDZ domains mediate protein-protein interactions; recognizing and binding to short, usually linear peptide epitopes found in various interacting partners (Hung and Sheng, 2002). PDZ domains bind to the extreme carboxyl termini of their binding partners which allows for a non-invasive way to mediate protein transport, localization, sorting, and spatial arrangement at particular locations, specifically the synapse (Kurakin et al., 2007). PDZ containing proteins can also dimerize with other PDZ domains. Due to the presence of multiple domains within a given protein and their ability to interact with a variety of partners, PDZ-domain containing proteins often act as scaffold proteins forming large multiprotein complexes. They have been implicated in variety of functions including endocytosis, surface delivery, and channel conductance (Kim and Sheng, 2004, Kurakin et al., 2007)

Classically, PDZ domains are sorted into two groups. Class I PDZ domains recognize and bind to the C-terminus of proteins containing the consensus sequence X-(S/T)-X- Φ-COOH, where X is any residue and Φ represents a hydrophobic residue. The second group of PDZ proteins, class II, bind to the consensus C-terminal sequence of X-Φ-X-Φ-COOH (Songyang et al., 1997). While these two groups are at present the most accepted way of categorizing PDZ domains, recent information has suggested differences and complexities within these two groups. For example, residues lying upstream of the minimal four C-terminal residues may play a significant role in determining specificity within the classical class I and class II groups (Kurakin et al., 2007).

An example of such a PDZ containing protein is PSD-95. PSD-95 is a member of the synapse-associated protein (SAP) family, and is encoded by PSD-95/SAP-90, PSD-93/chapsyn-110, SAP-102, and SAP-97 (Fujita and Kurachi, 2000). The characteristic c-terminal domain organization of these proteins is three N-terminal PDZ domains followed by a SRC homology 3 (SH3) domain and a guanylate kinase-like (GK) domain. PSD-95 is located approximately 12nm from the postsynaptic membrane (Peterson et al., 2003), placing it in prime location to influence postsynaptic membrane proteins such as receptors and ion channels. In heterologous cells PSD-95 has been shown to cluster NMDARs and Shaker-type K^+ channels (Kim et al., 1995). Clustering of NMDARs may occur through inhibition of receptor internalization- as studies in which removal of the C-terminal PDZ-binding motif of the NMDAR subunit led to increased internalization in neuronal cultures (Roche et al., 2001). In addition, PSD-95 can suppress Kv1.4 channel internalization (Jugloff et al., 2000). PSD95's influence over excitatory synapses has been further demonstrated through its effects on AMPAR mediated excitatory postsynaptic currents (ESPCs). Overexpression of PDS-95 leads to a potentiation of AMPAR mediated ESPCs while RNAi knockdown of PSD-95 results in ESPC suppression (Kim and Sheng, 2004).

PSD-95's ability to interact with ion channels provides an enticing and important way to affect neuronal excitability. Such evidence raises the question of whether other PDZ-domain containing proteins may interact with ion channels and similarly modulate neuronal excitability. Amongst the possible candidate proteins, Kir2 channels have shown to interact with SAP97 (Leonoudakis et al., 2004) and

PSD93 (Leyland et al., 2004) via their class I PDZ-binding motif (-ESEI) (Kim and Sheng, 2004). Kir channels are a family of inwardly rectifying potassium channels which play important roles in a variety of cellular functions including insulin release, vascular tone, neuronal firing rates, and synaptic inhibition. There are currently 7 known Kir subfamilies (Kir1-Kir7) (Ma et al., 2002). Kir3 or GIRK (G protein-gated inwardly rectifying potassium channels) are responsible for slow inhibitory postsynaptic potentials (sIPSP) and have been shown to be involved in addictive drug (Cruz et al., 2004) and alcohol response (Kobayashi et al., 1999). There are four mammalian GIRK subunits (GIRK1-GIRK4) which assemble in discrete combinations to form either homo- or heteroteramers (Stanfield et al., 2002). GIRK2c and GIRK3 both contain class I PDZ-binding motifs (-ESKV) which suggest that they may, like Kir2, interact with PDZ-domain containing proteins. In previous studies GIRK3 did not seem to interact with PSD-95 (Nehring et al., 2000). This finding is interesting given the sequence similarity between Kir2 (-ESEI) and GIRK3 (-ESKV). This finding suggests a higher level of discrimination than the classically defined four C-terminal residues. While GIRK3 failed to interact with PSD-95, a recent study demonstrated that these channels do in fact interact with another PDZ-domain containing protein- SNX27 (Lunn et al., 2007).

Through its PDZ domain SNX27 was shown to preferentially interact with GIRK2c and GIRK3 subunits. The ability to discriminate amongst the Kir subunits was shown to involve a PDZ domain sensitivity to the -1' position of the PDZ-binding motif (Lunn et al., 2007). Traditionally, the -1' position of class I PDZ-binding motifs

is considered variable. The ability of SNX27 to discriminate partners based on the -1' position suggests a new level of specificity. Currently SNX27 is the only PDZ-domain containing protein which also possesses a PX domain. This PX domain was shown to selectively bind to PI3P (Lunn et al., 2003), a phosphoinositide enriched in the early endosome. The early endosome is a component of the endocytic pathway and is critical in determining the fate of endocytosed membrane proteins. Upon internalization, proteins enter the early endosome and are targeted to the late endosome/lysosome for degradation or to the recycling endosome where they may be re-inserted into the membrane. Thus, the interaction between SNX27 and PI3P provides a potential mechanism for modulating GIRK3 trafficking. Indeed, GIRK3 and SNX27b colocalize with the early endosomal marker (EEA1) (Lunn et al., 2007). Furthermore, when GIRK3 co-assembles with GIRK1 to form functional channels GIRK3, GIRK1, and SNX27b all colocalize suggesting a functional interaction between the three proteins. In addition, HEK 293 cells transfected with GIRK3 and GIRK1 display significantly reduced GIRK1/3 currents in the presence of SNX27b. This reduction in current is attributed to decreased channel surface expression through targeted channel removal to the early endosome via SNX27b. SNX27's interaction, trafficking, and modulation of GIRK channels presents a new mechanism for controlling channel signaling and neuronal excitability (Lunn et al., 2007).

These recent results, taken in conjunction with the earlier data implicating SNX27 in methamphetamine addiction, provide an enticing mechanism for drug addiction and sensitization. Plausibly, drug administration causes an induction of

SNX27, which leads to an increase in internalization of GIRK channels and a reduction in current- resulting in a dis-inhibition and sensitization.

Further work is needed to exact the role SNX27 in sensitization. Critical to this venture is the localization of SNX27 in the brain. Distributional knowledge of the protein may give insight into its particular function. Utilizing a novel SNX27 antibody I hoped to elucidate SNX27 expression in the brain. A primary concern with any antibody is its reliability to interact specifically with the target antigen, SNX27. Thus, the first goal of my study was to characterize the novel antibody through western blotting and immunohistochemisty to demonstrate its specificity. Following antibody characterization, I wished to characterize the expression pattern of SNX27 in the brain, and lastly, to correlate SNX27 expression back to a functional significance involving GIRK channels.

CHAPTER 2: Determination of SNX27 Antibody Specificity

2.1 Testing SNX27 Bleeds for Specificity

Introduction:

Protein localization can be achieved through the use of specific antibodies created to target the protein of interest. Using peptides as immunogens, polyclonal antibodies can relatively quickly and easily be made against a nearly infinite number of targets (Howard and Bethell 2001). However, the usefulness of an antibody depends on its reliability to detect the target antigen. Poor recognition or low affinity for the antigen, along with impurities and low titers, can all negatively affect antibody specificity. Many factors can influence these properties, especially in polyclonal antibodies (Howard and Bethell., 2001). The immune response has a classical but not precise profile, making it difficult to predict the best time point for antiserum collection (Howard and Bethhell, 2001).

A polyclonal SNX27 antibody was generated against the first 19 residues of the N terminus of SNX27 (1-19). Since the N-terminus is conserved between the two splice variants (Kaji et al., 2003) (figure 2), it can detect both SNX27a and SNX27b, making it a pan-SNX27 antibody. To maximize the likelihood of collecting the best SNX27 antibody, antiserum was collected at multiple time points to sample across the immune response. Western blot analysis is an established method for testing antibody specificity (Howard and Bethell, 2001). Thus, I performed a western blot analysis to select the sample bleed which contained antibodies with the best affinity and specificity for SNX27.

Methods:

Transient Transfections

Human embryonic kidney 293 cells (HEK293) were cultured on 10cm dishes in a humidified 37°C incubator with 95% air and 5% CO_2. Cells were grown in DMEM with FBS (10%), penicillin (50µg/ml), streptomycin (50µg/ml), and glutamine (2mM). After plated cells reached 80% confluency media was changed and cells were transfected; 26µg of cDNA was used following the standard CalPhos™ Mammalian Transfection Protocol outlined in the Clontech Laboratories user manual. Transfections were incubated for 48h.

Western Blot

Protein was collected from HEK293 cells transiently transfected as described earlier. Protein extraction was carried out using the Trizol® method (Invitrogen, Carlsbad). The resulting protein pellets were stored at -20°C in 100% ethanol. Right before use, pellets were re-suspended in 1% SDS and 2-mercaptoethanol (10%). Suspended protein (20-25µl) was load into a 12% SDS-page gel and ran out at 110V. Protein was then transferred to a nitrocellulose membrane at either 80V for 3h or 60V overnight. Membranes were blocked in 5% milk in PBS solution for 2h at room temperature or overnight at 4°C, under constant agitation. Blocking solution was rinsed off by three 15m PBS rinses. Rinses were followed by a 2h incubation with primary polyclonal rabbit anti-pan-SNX27 antibody diluted to 1:5000 in PBS with 5%

milk under constant agitation. Membranes were then rinsed with PBS and probed with chicken anti-rabbit-HRP secondary antibody (Santa Cruz, Sc-2961) diluted to 1:200 in PBS with 5% milk for 1h with constant agitation at room temperature. Following a final PBS rinse, membranes were briefly exposed to SuperSignal ECL reagents (Pierce, Rockford). The resulting luminescence was detected with BioMax XAR film (Eastman Kodak, Rochester).

Antibodies

Pan-SNX27: Polyclonal rabbit anti-panSNX27 raised against the N-terminus of SNX27 (1-19)

Antibodies were aliquoted into small volumes of either 5µl or 10µl volumes and stored at -80°C until used.

Results:

All SNX27 bleed dates, except February, detected protein collected from HEK293 cells transfected with SNX27b. The detected protein was 60kDa, the expected size for SNZ27b (figure 3). In the presence of protein collected from HEK293 transfected with SNX27b-YFP the detected protein shifted in molecular weight to ~90 kDa (figure 3). No protein was detected in lanes containing protein collected from untransfected HEK293 cells (NT). No protein was detected when protein samples were probed with normal rabbit serum (NRS) (figure 3).

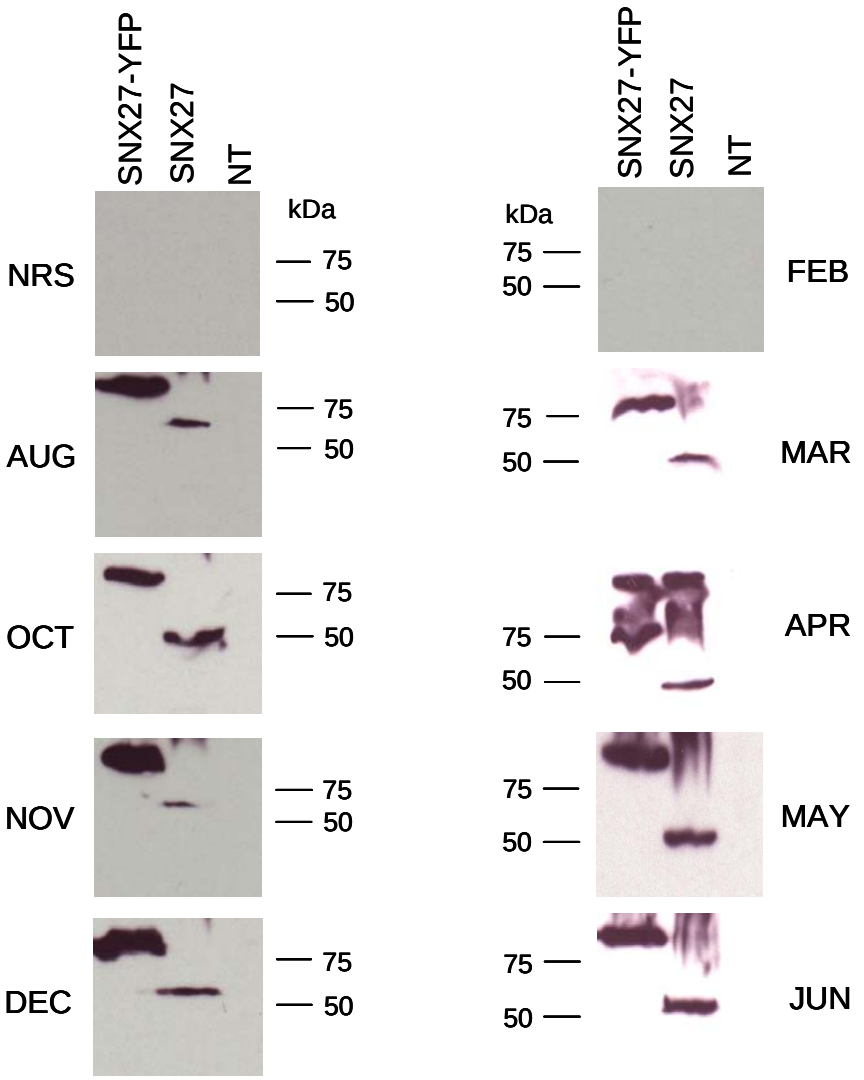

Figure 3. Specificity of various SNX27 bleed dates. HEK293 cells were transfected with SNX27 or SNX27-YFP cDNA. Protein was isolated from whole cell lysate preps using the Trizol protocol and loaded onto a 12% gel for SDS-PAGE. Proteins were transferred to a nitrocellulose membrane and probed with anti-SNX27 rabbit polycolonal antibody from various bleed dates or with normal rabbit serum (NRS). SNX27 antibody from most bleed dates displayed strong specificity at the appropriate 60kda band size and the corresponding shift for SNX27-YFP. NRS did not recognize SNX27 protein. Protein samples from untransfected (NT) HEK293 cells lacked antibody recognition.

Discussion:

The August bleed proved to be the best bleed, with consistent and strong detection of the protein with very little background. Thus, this bleed was selected for future SNX27 antibody work. Still, western blotting demonstrated that nearly all SNX27 antibody samples had decent specificity. The appropriate band shift for SNX27-YFP further ensured its specificity. The lack of protein recognized by NRS demonstrated that the recognized protein was not a non-specific interaction with a component found in rabbit serum.

The peptide used to generate the SNX27 antibody seemed to prompt a robust and specific immunological response as nearly all samples strongly detected SNX27. Oddly, antibody recognition occurred early and late in the immunization protocol, with loss of recognition during the middle (February). This could be the result of typical fluctuations in antibody concentrations that tend to peak, decrease, and resurge depending on the immunization protocol (Howard et al., 2001).

CHAPTER 3: SNX27 Distribution in Brain and Peripheral Organs

3.1 Regional Expression of SNX27

Introduction:

With demonstration of SNX27 antibody specificity I wished to examine gross SNX27 distribution in the body. Several SNX27 binding partners had already been identified including diacylglycerol kinase ζ, CASP, and 5-HT_{4a} (MacNeil et al., 2007, Rincón et al., 2007, Joubert et al., 2004). A previous study also demonstrated that SNX27 mRNA was expressed in a variety of organs and brain regions (Kaji et al., 2003). This ubiquitous expression, along with its multiple binding partners, hints at the importance of SNX27 function. To test if mRNA expression translated into similar protein expression I performed a regional western blot to probe for SNX27 expression in various brain regions and peripheral organs.

Additional Methods:

Animal Protein Collection

Protein samples used in multi-tissue and brain region westerns were collected from PD0 rats. Rats were decapitated and the appropriate tissues removed and homogenized using the Trizol® protocol (Invitrogen, Carlsbad).

Results:

SNX27 protein was detected in a variety of organs and brain regions. Most peripheral organs showed the presence of SNX27 (figure 4). SNX27 protein was not

detected in the heart and lungs. In the brain, with the exception of the thalamus, all regions demonstrated SNX27 protein expression. The presence of loaded protein was confirmed through the detection of actin.

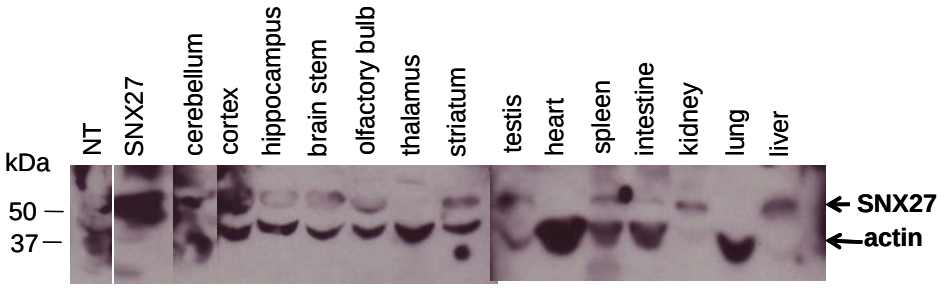

Figure 4. Multi-region expression of SNX27. Protein samples from various rat organs and brain regions were homogenized and collected for SDS-PAGE. Nitrocellulose membrane was probed with SNX27 antibody. Most regions, with the exclusion of the heart, lung, and thalamus, showed the presence of SNX27.

Discussion:

SNX27 was found to be expressed in nearly all peripheral organs and brain regions. This ubiquitous expression correlates well with mRNA data. If indeed SNX27 is important in drug sensitization then it would be expected to be present in structures involved in this process. A wide range of brain structures have been implicated in sensitization with the list continually growing to include the nucleus accumbens, striatum, ventral tegmental area, prefrontal cortex, amygdala, neocortex, and hippocampus (Kaji et al., 2003, Hyman et al., 2006) (figure 1). Indeed SNX27 was found to be in many of these areas. In addition, SNX27 has been shown to interact with diacylglycerol kinase ζ and CASP; and to modulate their trafficking (MacNeil et al., 2007, Rincón et al., 2007). Both these binding partners are enriched

in lymphocytes (MacNeil et al., 2007, Rincón et al., 2007) which may explain the detection of SNX27 in many organs.

CHAPTER 4: SNX27 Cortical and Hippocampal Immunohistology Using DAB

4.1 Determination of SNX27 Specificity in DAB:

Introduction:

To better ascertain SNX27's possible neuronal function I decided to look at its cellular distribution in the brain. To do this I chose the immunohistochemical assay using the standard 3,3'diaminobenzidine (DAB) staining protocol. However, before beginning such experiments I tested for SNX27 antibody specificity in DAB staining. While the antibody showed specificity via western blotting, antibody specificity can vary depending on the assay (Howard and Bethell, 2003). Ideally the ablation of staining in a knockout animal provides the most convincing demonstration of immunohistochemical antibody specificity (Saper and Sawchenko, 2003). However, such an animal was not available for SNX27 antibody testing. Another widely acceptable test of specificity is a competition/pre-absorption test (Saper and Sawchenko, 2003). In a competition study primary antibody is pre-incubated with its cognate peptide before application to tissue. During this pre-incubation the antibody should specifically bind to the peptide and thus be "occupied" and unable to interact with the antigen in tissue. By using a peptide gradient a gradual reduction in staining should be achieved. Staining that is decreased through competition can be designated specific; whereas, unaffected staining is non-specific. Thus, I performed a competition study to examine SNX27 specificity in DAB staining.

Additional Methods:

Animals

Adult male Sprague-Dawley rats (250-300g) were used in all experiments. Rats were housed two or three per cage in environment controlled rooms, and had free access to water and food at all times. All animal handling and procedures were approved by the Institutional Animal Care and Use Committee at the Salk Institute.

Tissue Preparation

Rats were anesthetized with chloral hydrate (350mg/kg) administered intraperitoneally. Anesthetized rats were then perfused through the ascending aorta with saline followed by 600-700ml of 4% paraformaldehyde in 0.1 M borate buffer (pH 9.5 (n=12) or 7.4 (n=3)) at 10°C or Zamboni (n=1); fixative was administered for approximately 20m using a peristaltic pump. Fixed brains were then postfixed for 2h in the same fixative used for perfusion. Brains were transferred to a 15% sucrose solution and stored overnight for cyroprotection. The following day the brains were frozen in dry ice and sectioned into series of 30µm thick coronal sections using a freezing microtome. The free floating sections were then stored in antifreeze at -20°C until immunohistochemical processing.

DAB Immunohistochemistry:

Antifreeze was removed from the free-floating coronal sections by KPBS washes. Engodenous peroxidases were quenched by washing in 0.3% hydrogen

peroxide for 10m. Sections were then incubated in rabbit polyclonal anti- SNX27 antibody or pooled normal rabbit serum (NRS). Primary antiserum was diluted to the appropriate concentration in KBPS with 0.03% Triton and 2% normal goat serum as a blocking agent. Sections were incubated in primary antibody for 48h at 4°C with constant agitation. After incubation sections were rinsed 3 times in KPBS and incubated for 1h in secondary goat anti-rabbit biotinylated antibody (Vector BCA, Burlingame) at a dilution of 1:200 in KPBS with 0.03% Triton. Secondary incubation was followed by 3 KPBS rinses and a 1h incubation in Avidin Biotin Peroxidase Complex (ABC) (Vecstastain Elite® kit) (Vector, Burlingame). Antibody localization was visualized by section exposure to DAB and subsequent processing. Stained sections were then mounted onto gelatin coated glass slides, dehydrated through graded alcohols, cleared in xylene, coversliped, and imaged.

Competition Studies*:*

SNX27 antibody was pre-incubated overnight at 4° C under constant agitation with its cognate peptide. SNX27 was blocked at a concentration of 1:3000 with varying peptide concentrations of 1.0mg/ml, 0.5mg/ml, 0.1mg/ml, 0.05mg/ml, or 0.01mg/ml. Antibody in control sections were pre-incubate without peptide but were stored at 4° C under constant agitation overnight. Following pre-incubation DAB staining was conducted using the previously described protocol.

DAB Imaging

Slides were imaged using light microscopy on a Leica DMRP microscope. Images were captured using an attached Hananatsu C4742-95 Orca II camera and imported into Improvision Open Lab software. Exposure parameters were either auto-optimized by Open Lab or manually adjusted to be consistent with previous exposures. Files were then exported as .tiff files.

Results:

Sections stained with antibody blocked at the lowest peptide concentration (0.01mg/ml) had a nearly identical cortical and hippocampal staining pattern compared to control sections (figure 5A). Diffuse staining was seen throughout the hippocampus and cortex, with intense cell layer and cell body labeling seen in the hippocampus and cortex respectively (figure 5B). Strong glial staining was seen in both structures. As the concentration of the peptide increased, SNX27 staining noticeably decreased. At the highest concentration (1.0mg/ml) nearly all intense staining in the cell layers and cell bodies was abolished in both the hippocampus and cortex (figure 5A, B). Glial staining in both regions was completely attenuated (figure 5B). Equal reduction in staining was not seen for non-cellular regions of the hippocampus and cortex. In these areas a diffuse background stain remained even at the highest blocking concentration (figure 5A).

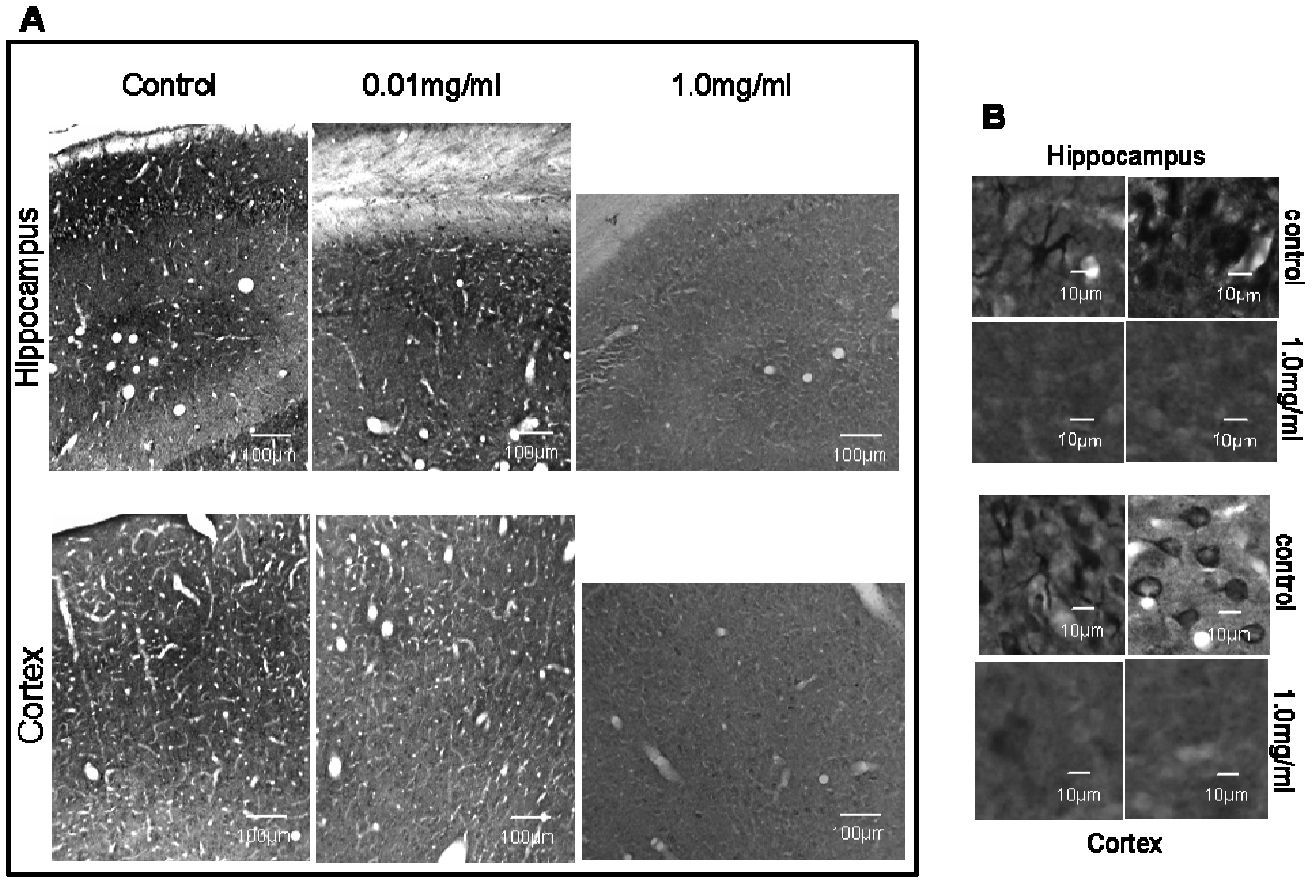

Figure 5. SNX27 antibody specificity in DAB. SNX27 antibody was pre-incubated with different concentrations of its cognate peptide. Rat coronal sections were then probed using the pre-incubated antibody and processed via DAB. At 0.01mg/ml SNX27 staining resembles that ofcontrol sections **(A)**. At 1.0mg/ml staining is greatly reduced in both the hippocampus and cortex **(A)**. Zooms of control and 1.0mg/ml sections display the reduction in glial and cell body labeling **(B).**

Discussion:

DAB analysis resulted in a partially specific signal from the SNX27 antibody. At the highest peptide concentration not all staining was blocked. However, staining in the cellular layers of the hippocampus (DG, CA3, CA1) and cortex were nearly completely attenuated. Thus, it is likely that staining in these regions represented specific SNX27 staining. The diffuse background staining that did not decrease at the highest peptide concentration was non-specific background staining. This background staining is problematic because it may interfere with specific staining in those regions. Such high background noise may prevent a true signal from being detected. Thus several attempts were made to reduce this background staining.

This non-specific staining could be the result of methodological inadequacies. Fixation of tissue cross-links proteins and can occlude them from being available for interaction with an antibody (Howard and Bethell, 2001). In addition, the harsh treatment of the tissue can affect important tertiary structures needed for the antibody to recognize the protein (Howard and Bethell, 2001). Changing the fixation protocol may thus allow the antibody to more readily access and bind the antibody. I ran several changes in protocol to try to eliminate background staining. I varied the pH of the fixative, changed the fixative from paraformaldyhde to zamboni, and varied both the perfusion and postfix times. None of these changes decreased the non-specific staining background.

4.2 Cortical and Hippocampal SNX27 Expression in DAB:

Introduction:

Earlier *in situ* studies demonstrated a wide range of SNX27 mRNA expression, with detection in nearly all brain structures (Lunn et al., 2007). Noticeably higher levels of expression occurred in the medial septum, cranial nerve motor nuclei, cerebellar cortex, and hippocampus (Lunn et al., 2007). I wished to see if mRNA expression could be correlated into a similar SNX27 protein expression in areas implicated in drug addiction. Both the cortex and hippocampus have been suggested to be important in addiction (Hyman et al., 2006, Jones et al., 2005) and both structures demonstrated high levels SNX27 mRNA (Lunn et. al., 2007). Thus, I looked at SNX27 expression in the cortex and hippocampus.

Methods:

See previous sections

Results:

Cortex

SNX27 antibody labeled cells throughout the cortex (figure 6A). These cells displayed perisomatic staining which was slightly punctate in nature (figure 6B). Significant glial staining was also detected throughout the cortex (figure 6C). Both

cell body staining and glial staining were seen in the presence of a diffuse background staining (figure 6A). Sections incubated without primary antibody (no-primary) showed no staining. A high degree of staining was seen throughout the cortex when normal rabbit serum (NRS) was used as the primary antibody instead of SNX27 (figure 6A). NRS produced diffuse staining in the cortex; with more intense staining in a laminar layer of cells (figure 6A, B). Glia staining was absent in NRS (figure 6C).

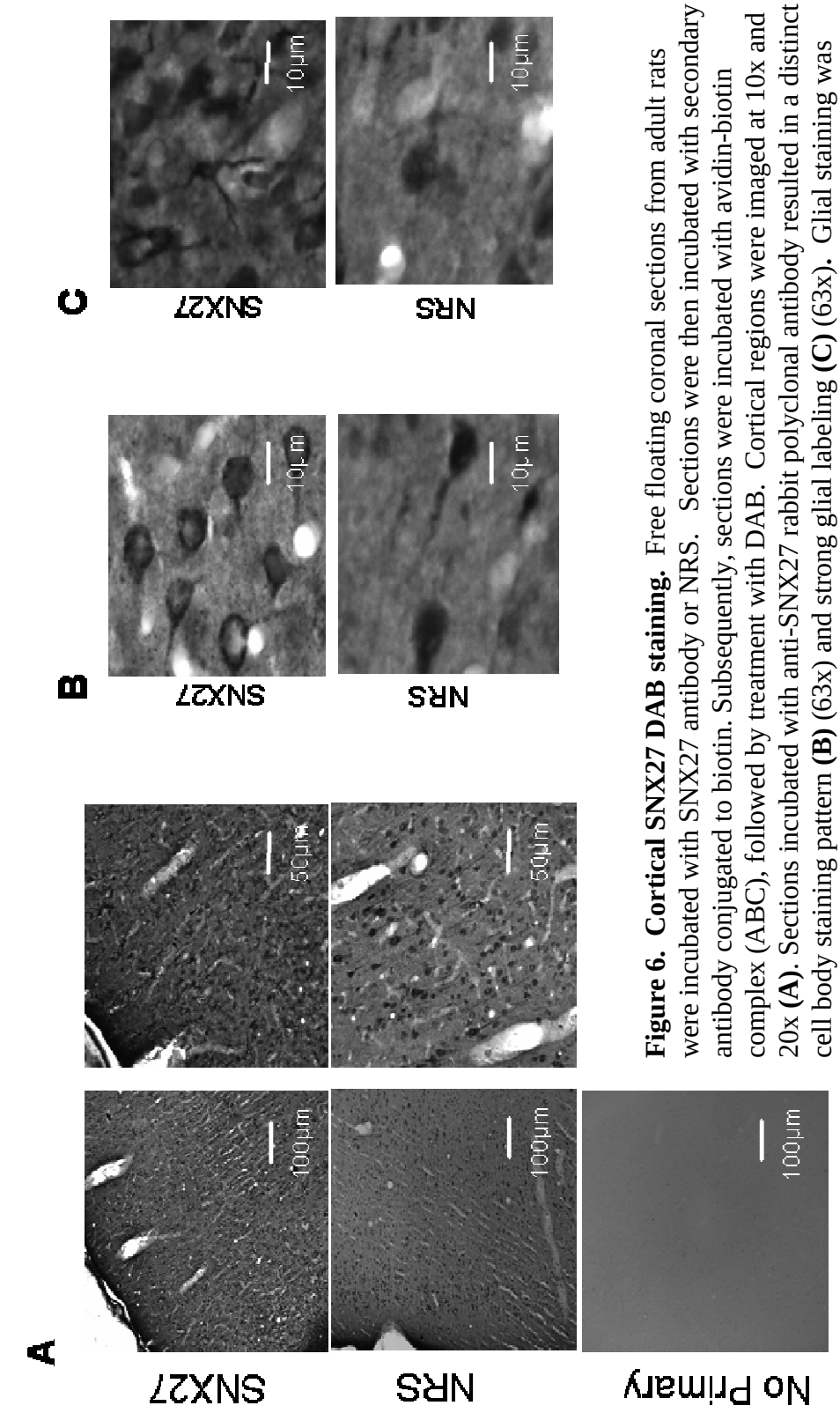

Figure 6. Cortical SNX27 DAB staining. Free floating coronal sections from adult rats were incubated with SNX27 antibody or NRS. Sections were then incubated with secondary antibody conjugated to biotin. Subsequently, sections were incubated with avidin-biotin complex (ABC), followed by treatment with DAB. Cortical regions were imaged at 10x and 20x **(A)**. Sections incubated with anti-SNX27 rabbit polyclonal antibody resulted in a distinct cell body staining pattern **(B)** (63x) and strong glial labeling **(C)** (63x). Glial staining was absent in sections incubated with NRS **(C)** and cell body labeling was significantly different from that of SNX27 **(B)**. Diffuse background staining was seen in both the presence of SNX27 and NRS. Slices incubated without primary antibody did not display staining **(A)**.

Hippocampus

Hippocampal staining had significant background (figure 7A). Glial staining was present in all areas of the hippocampus, especially in the stratum radiatum of the CA1 (figure 7A, C). The cellular layers of the CA1, CA3, and DG showed intense cell body staining (figure 7A, B). Within these layers, distinct cellular labeling was difficult to ascertain as cell morphology in hi-resolution images was inconclusive (figure 7B). NRS faintly labeled these cells in a similar fashion, but staining was much less intense (figure 7A, B). Glial staining was absent in NRS (figure 7A,B). No staining was seen with no-primary (figure 7A).

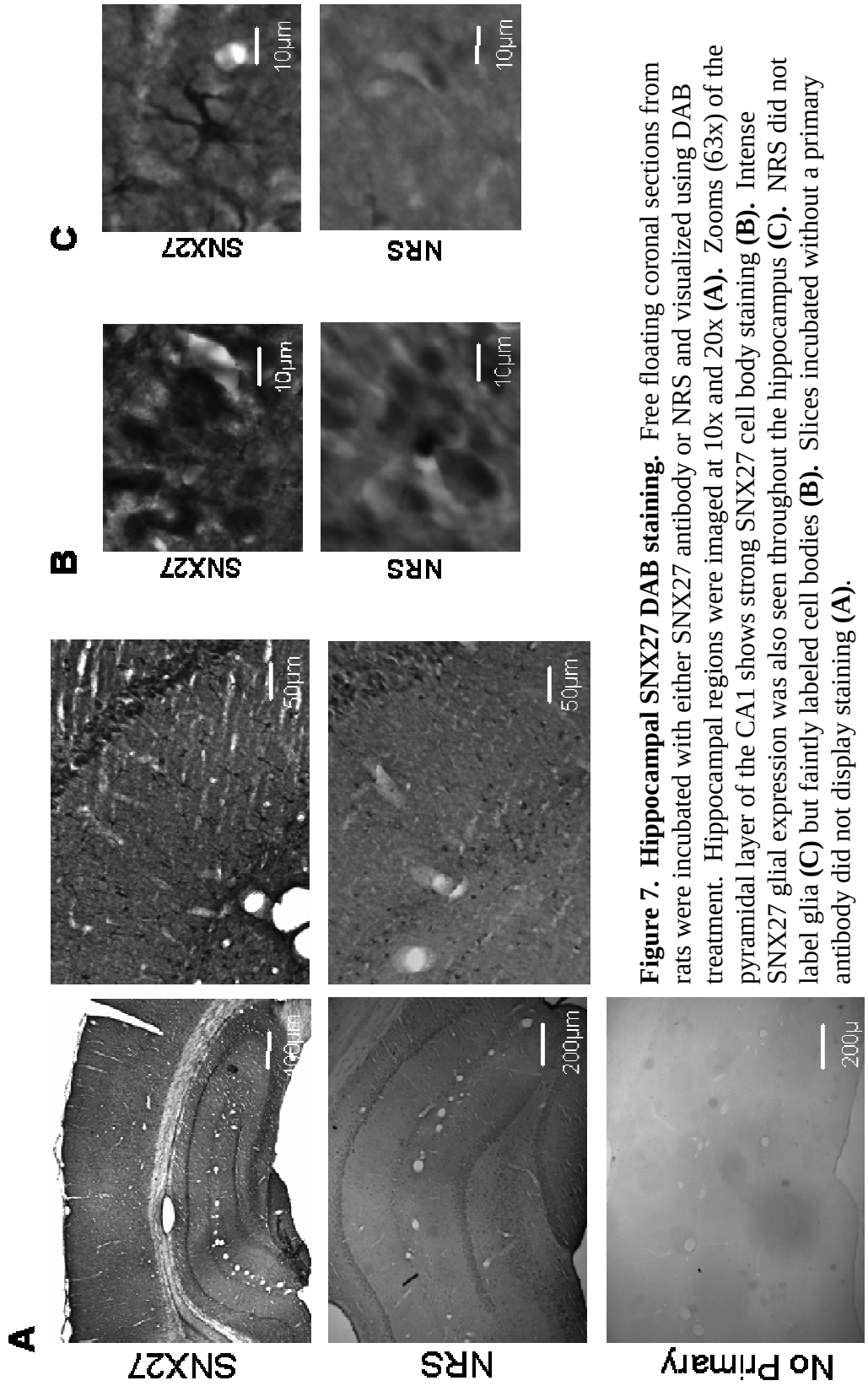

Figure 7. Hippocampal SNX27 DAB staining. Free floating coronal sections from rats were incubated with either SNX27 antibody or NRS and visualized using DAB treatment. Hippocampal regions were imaged at 10x and 20x **(A).** Zooms (63x) of the pyramidal layer of the CA1 shows strong SNX27 cell body staining **(B).** Intense SNX27 glial expression was also seen throughout the hippocampus **(C).** NRS did not label glia **(C)** but faintly labeled cell bodies **(B).** Slices incubated without a primary antibody did not display staining **(A)**.

Discussion:

SNX27 protein was found to be ubiquitously expressed in a variety of brain structures, notably the cortex and hippocampus. Strong glial staining was seen in both structures. The glial staining seen corroborates early studies in which SNX27 colocalized with the glial marker, GFAP, in hippocampal dissociated cultures (Nassirpour and Slesinger, unpublished). The morphology of these glia showed some variation. Certain cells were composed mainly of branched processes, with little cytoplasm; while others appeared to be larger in size and more radial-like. Such staining suggests a heterogeneous population of microglia and astrocytes.

The perisomatic pattern in cortical cells suggests localization of SNX27 to the cell membrane or localization in the cytoplasm directly under the membrane. Many sorting nexins have shown targeting to PI3P enriched membranes in the endocytic pathway. SNX3 colocalizes with EEA1 (Xu et al., 2001) and SNX1 displays discrete punctate expression overlapping with early endosomal markers (Zhone et al., 2002, Cozier et al., 2002, Kruten et al., 2001). Cytoplasmic labeling could be the result of PX-mediated localization of SNX27 to the early endosomes via an interaction with PI3P (Seet et al., 2006). Studies have already demonstrated that SNX27 is targeted the early endosome where SNX27 colocalizes with EEA1 in a punctate fashion (Lunn et al., 2007). Supporting early endosomal expression, the perisomatic staining was slightly punctate in nature which would be expected in early endosome localization.

SNX27 expression in the hippocampus was difficult to characterize at the cellular level due to distorted cell morphology, likely the result of harsh tissue

handling. Staining pattern within the various layers could be discerned, with intense staining seen only in glia and the cell bodies of the principle cells in the DG, CA3, and CA1. However, similar, albeit, fainter staining was seen in NRS. This similarity in staining raised the concern that SNX27 labeling may be non-specific. The lack of staining in no-primary sections ruled out the possibility that non-specific staining was the result of a promiscuous secondary antibody.

There are several possibilities which could explain the high NRS staining. Firstly, the NRS used in the experiment was pooled serum; coming from rabbits different than those in which the antibody was generated. It is possible that these rabbits themselves were undergoing an immunological response during the time of collection which generated copious amounts of antibodies. A better control which replaces NRS is pre-immune serum. This serum comes specifically from the animal in which the antibody is generated and is collected right before immunization. Thus, both the pre-immune serum and the antiserum are more likely to have similar non-specific antibody profiles (Saper and Sawchenko, 2003). Secondly, NRS may contain antibodies with high affinity for their antigens. If the SNX27 antibody had a low affinity for SNX27, it is conceivable that antibodies found in rabbit serum could obscure nearly all specific SNX27 antibody signal.

CHAPTER 5: SNX 27 Hippocampal

Immunohistology Using Fluorescence

5.1 Determination of SNX27 Fluorescent Specificity:

Introduction:

The non-specific staining seen using DAB was higher than optimal. In an attempt to optimize staining I decided to switch from DAB to fluorescent staining. As a change in protocol, such as the switch from DAB to fluorescence, may affect the levels of non-specific interactions I decided to perform another competition study to test for SNX27 specificity in immunoflourescence.

Additional Methods:

Fluorescent Immunohistochemistry

Free floating coronal sections were rinsed in KBPS and incubated for 48h in the appropriate combination of primary antibodies diluted in KPBS with 0.3% Triton and 2% normal goat serum: SNX27 (1:1000), GFAP (1:2000, Advanced ImmunoChemical Inc 031223), NeuN (1:5000, Chemicon International MAB377), MAP-2 (1:200, Sigma M4403), PSD95 (1:2000, ABR MA1-045). After primary incubation, sections were rinsed in KPBS and then incubated for 2h using fluorophore-conjugated secondary antibodies (1:200, Molecular Probes): rabbit-alexa488 (A-21206), mouse-alexa647 (A-21463), guinea pig (A-21450). After secondary incubation, sections were rinsed in KPBS, mounted on gelatin coated slides, coversliped, and imaged.

Fluorescent Imaging

Slides were imaged by laser confocal microscopy on the Zeiss LSM5 Pascal under 10x, 25x, 40x(oil immersion), and 63x(oil immersion) lenses using HeNe and argon lasers. For comparison of control and antibody staining, parameters including gain, pinhole size, and % transmission were held constant.

Results:

SNX27 staining was a gradually abolished with increasing peptide concentration (figure 8A). At the highest peptide concentration, 1.0 mg/ml, a significant amount of SNX27 staining was blocked. In particular, nearly all glial staining was blocked. Perisomatic staining was reduced, though not abolished. NRS staining was not affected by the introduction of blocking peptide at 1.0 mg/ml (figure 8B).

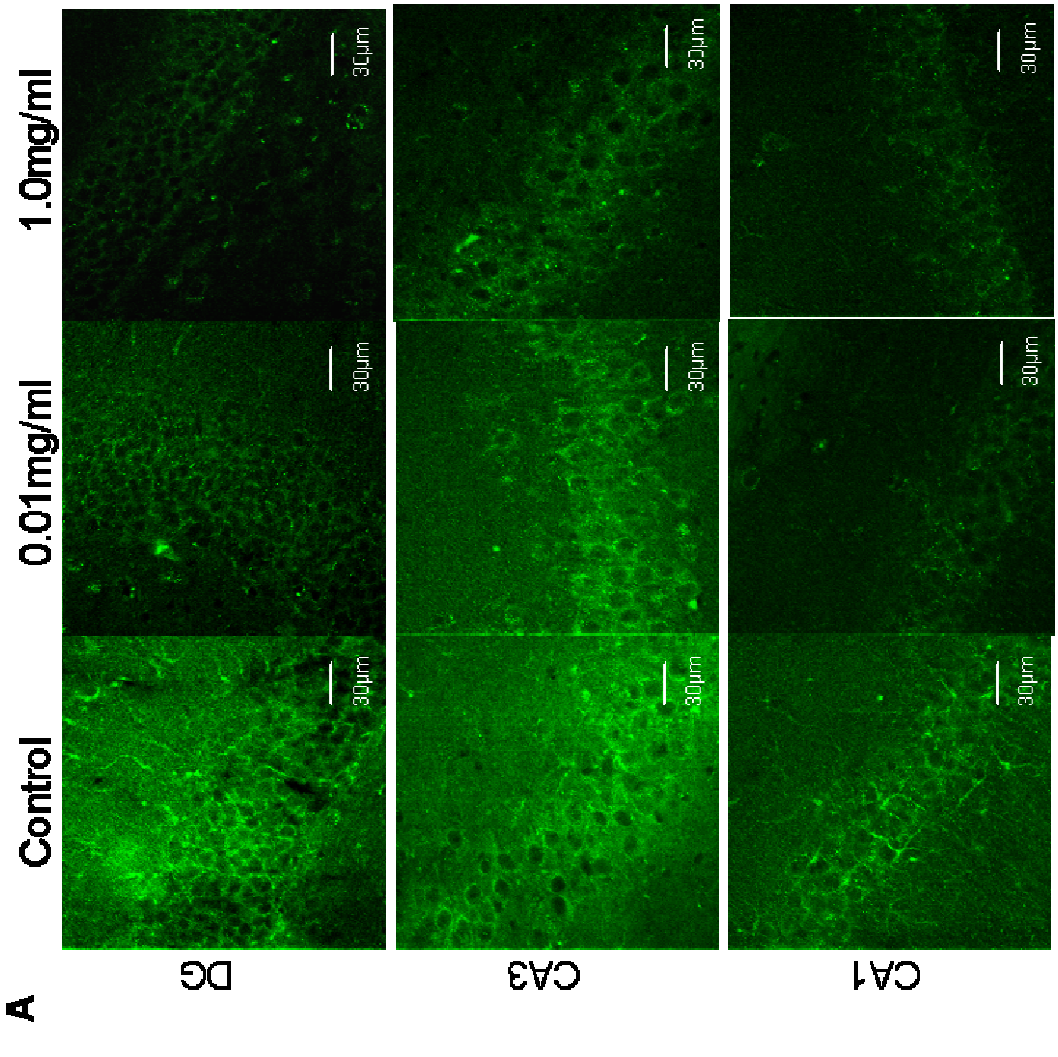

Figure 8. SNX27 specificity in immunofluorescence. SNX27 antibody was pre-incuabeted with 0.01mg/ml or 1.0mg/ml of its cognate peptide. Sections probed with the pre-incubated peptide showed a decrease in, but not complete ablation of staining (**A**). This reduction was not seen in NRS pre-incuabted with peptide at 1.0mg/ml (**B**).

Discussion:

Reduction in staining with increasing peptide concentration demonstrated that SNX27 fluorescent staining was specific. However, it appeared to be only partially specific as low levels of staining remained even at the highest blocking concentration. Staining was not equally attenuated in all areas of the hippocampus. Glial labeling was most certainly abolished by peptide blocking; indicating that SNX27 antibody specifically labels glia. However, such a distinct ablation in other areas and cell types was not seen. In the cellular layers some of the perisomatic staining remained. Puncta within the perisomatic labeled area decreased, though some remained. This suggests the punctate staining was partially specific. The presence of artifactual puncta in the cellular layers may be due to the high density of cells in these areas (O'Keefe et al., 1978) which can cause secondary antibody to non-specifically deposit in these regions. NRS was pre-incubated at 1.0mg/ml to test the possibility that the peptide was blocking an antigen found in rabbit serum rather than SNX27 antibody. Introduction of the peptide to NRS did not augment NRS staining, demonstrating that blocking was specific to the SNX27 antibody.

5.2 SNX27 Hippocampal Expression

Introduction:

The hippocampus is an integral component of learning (MRC, 2003). Extensive studies of its pathways gave rise to the first models of long term potentiation (LTP) and long term depression (LTD) (MRC, 2003). Recent models have incorporated the hippocampus into the mechanistic explanation of drug sensitization (Jones et al., 2005, Hyman et al., 2006). The incentive-sensitization theory of addiction postulates that repeated drug exposure can sensitize both behavioral and neuronal circuits that assign incentive salience to drugs and drug related cues (Robinson et al., 1993). This theory hinges on associative learning that links specific cues to drug wanting and seeking (Hyman et al., 2006). Both LTP and LTD are associative and synapse specific which makes them good candidate mechanisms for experience-dependant learning, which is essential to sensitization (Jones et al., 2005, Martin et al., 2000, Malenka et al. 2004). Thus, the neuronal circuits of the hippocampus likely play an important role in sensitization. The fact that SNX27 expression is modulated in sensitization (Kaji et al., 2003) suggests that it may perform an important function in hippocampal mediated sensitization. I decided to limit my survey to the hippocampus so that hippocampal SNX27 expression could be more thoroughly examined.

The hippocampus has a highly defined structure. The basic structure of the hippocampus is two interlocking c-shaped structures: the dentate gyrus (DG) and the

hippocampus proper, which is comprised of CA1-CA4 (O'Keefe et al., 1978) (figure 9A). There are two types of excitatory principle neurons found in the hippocampus: granular cells and pyramidal neurons, which are found in the DG and hippocampus proper respectively. There are also inhibitory interneurons in each of these regions (Freund et al., 1996). The DG is composed of three layers- the *granular* layer which consists of densely packed granule cells, the *moleculare* layer formed from the apical dendrites of the granule cells and their afferent connections, and the *polymorph* layer which contains interneurons and granule cell axons that being to bundle into mossy fiber tracts (O'Keefe et al., 1978) (figure 9B). Like the DG the hippocampus proper has a highly organized structure. The hippocampus proper is further broken down into four fields: cornu ammonis (CA)1-4. The CA4 is a poorly defined region of scattered cells in the polymorph layer of the DG. The other CA regions are organized into 5 layers. Starting with the outermost layer- the *alveus* contains axons from pyramidal neurons, the *stratum oriens* contains interneurons and the basal dendrites of the pyramidal cells, pyramidal neurons comprise the *pyramidale* layer, and their apical dendrites propagate through the *stratum radiatum* and *stratum moleculare* layers of the hippocampus. The CA3 region has an addition layer, *stratum lucidum*, which lies between the stratum pyrmidale and stratum radiatum (O'Keefe et al., 1978) (figure 9B).

The layers of the hippocampus (DG, CA1, CA3) form a closed tri-circuit which relays information, predominately unidirectional, from the DG to the CA3 to the CA1. Input from the entorhinal cortex signals to the granular cells of the DG,

which synapse onto the pyramidal neurons of the CA3 through the mossy fiber pathway. CA3 neurons have two main output tracks, one to the CA1 via Schaffer collaterals, and one that projects out of the hippocampus to the lateral septum. CA3 can also back project to the DG. The CA1 is the main output signal of the hippocampus, signaling to the subiculum and onto the entorhinal cortex (O'Keefe et al., 1978). In addition to this tri-circuit, a variety of inhibitory GABAergic interneurons throughout these layers modulate the action of the principle cells (Freund et al. 1996).

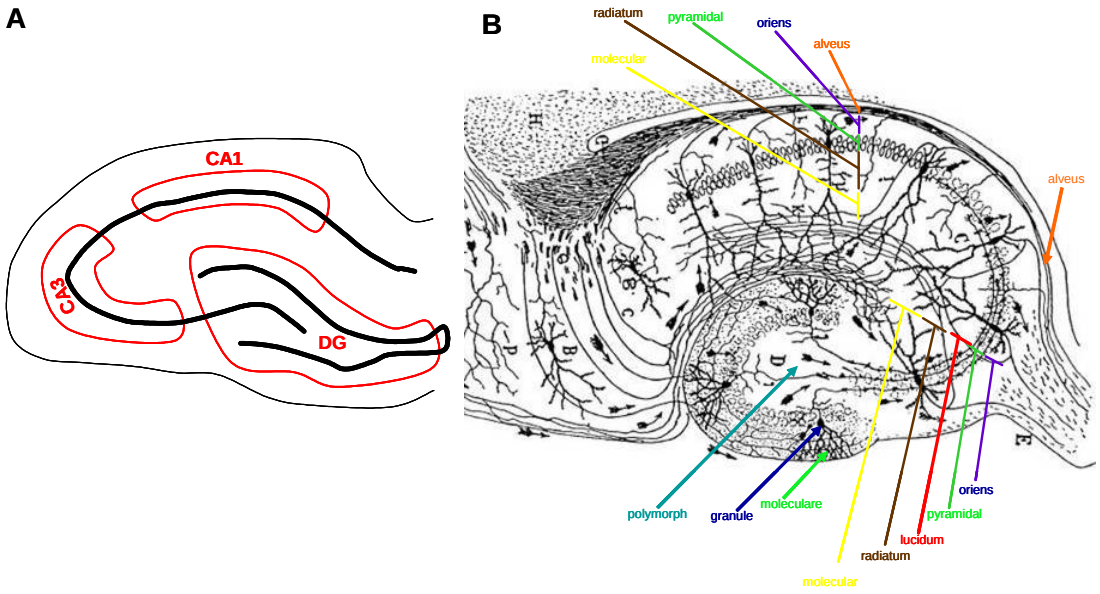

Figure 9. Anatomy of the hippocampus. The hippocampus is comprised of two interlocking c-shaped cortexes. The circuitry of the hippocampus involves three main layers, the dentate gyrus (DG), cornu ammonis 3 (CA3) and cornu ammonis 1 (CA1) **(A)**. The DG is comprised of three layers: *moleculare*, *granule* and *polymorprh*. The CA3 and CA1 have five main layers: *alevus*, *stratum oriens*, *pyramidale*, *stratum radiatum*, and *moleulare lacunosum* **(B)**. Image adapted from Takahashi D.K. (2006)

Methods:

See previous sections

Results:

Dentate Gyrus

Diffuse background staining was seen throughout the DG. Perisomatic staining was seen outlining the cell bodies within the granular layer (figure 10). Perisomatic staining was diffuse with some concentrated puncta. Granule cells were devoid of nuclear staining (figure 10). Dendritic-like staining was also seen in the granular layer with projections occasionally extending into the moleculare layer of the DG (figure 10). Glial staining was present in all three layers the DG (figure 10). NRS produced diffuse staining in all areas of the DG with intense staining in granular layer (figure 10). Glial staining was absent in NRS. No staining was seen in no-primary controls (figure 10).

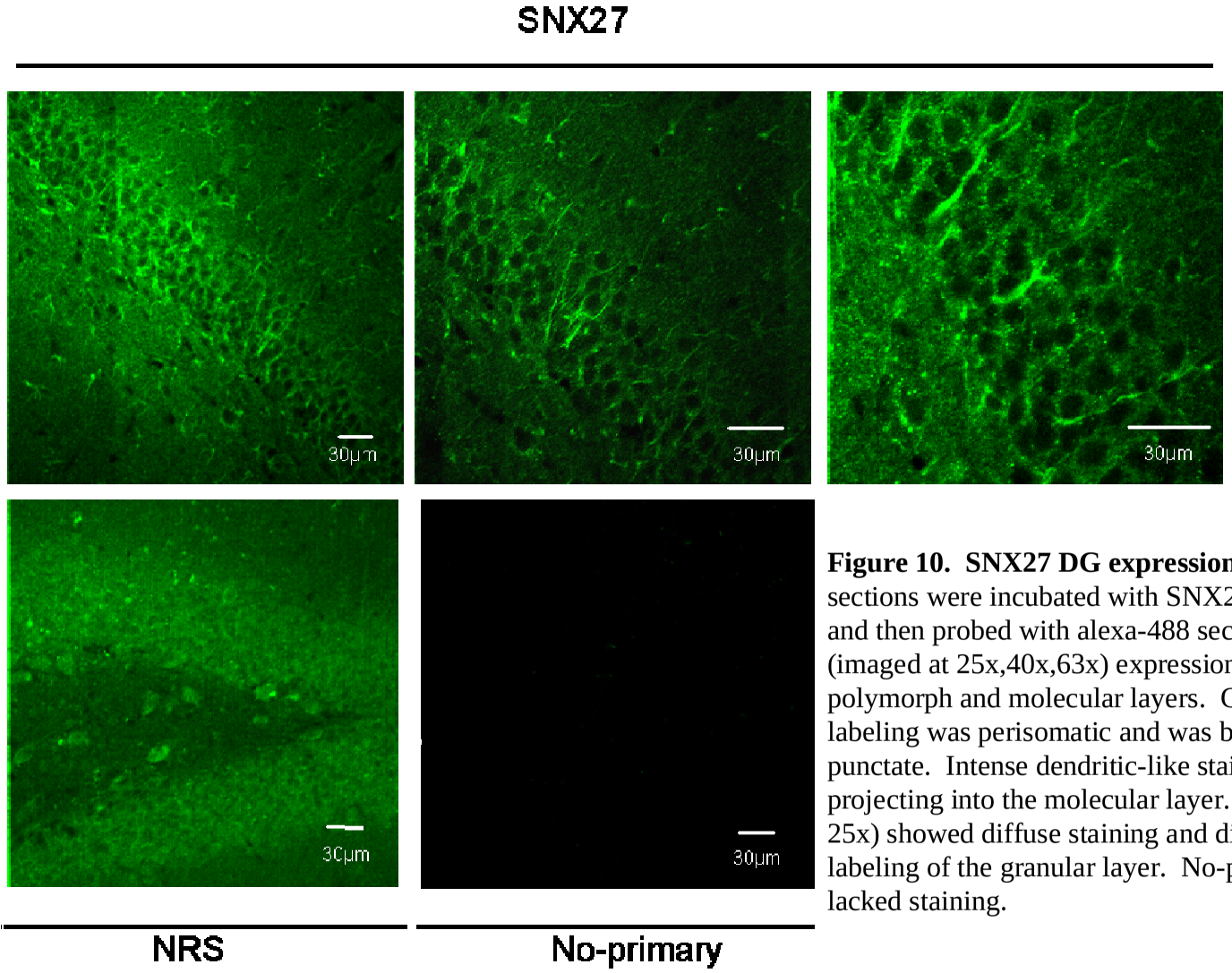

Figure 10. SNX27 DG expression. Free floating sections were incubated with SNX27 antibody or NRS and then probed with alexa-488 secondary. SNX27 (imaged at 25x,40x,63x) expression was diffuse in polymorph and molecular layers. Granular cell labeling was perisomatic and was both diffuse and punctate. Intense dendritic-like staining was seen projecting into the molecular layer. NRS (imaged at 25x) showed diffuse staining and distinct cell body labeling of the granular layer. No-primary sections lacked staining.

Cornu Ammonis 3

Pyramidal CA3 neurons were perisomatically labeled. Labeled cells were scattered and occurred at a low density (figure 11). Cell staining was polarized, with increased expression seen at the apical edge of the soma, often punctuate in nature (figure 11). Dendritic-like projections were absent in the CA3 but glial staining was present in the stratum oriens, pyramidale, and stratum lucidum (figure 11). Both SNX27 and NRS displayed background staining. NRS labeled pyramidal cell bodies (figure 11). No-primary sections had very limited amounts of staining.

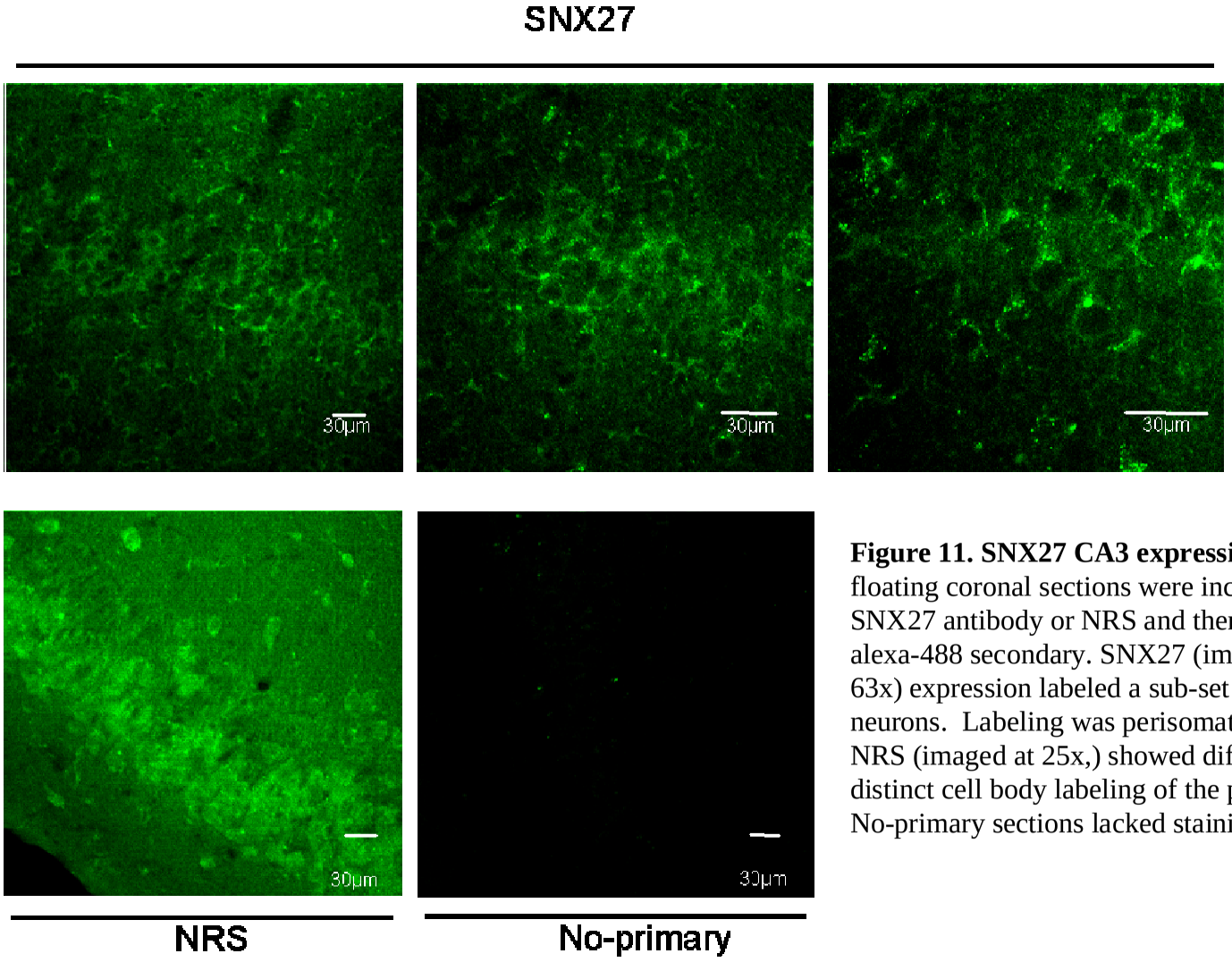

Figure 11. SNX27 CA3 expression. Free floating coronal sections were incubated with SNX27 antibody or NRS and then probed with alexa-488 secondary. SNX27 (imaged at 25x, 40x, 63x) expression labeled a sub-set of pyramidal neurons. Labeling was perisomatic and punctate. NRS (imaged at 25x,) showed diffuse staining and distinct cell body labeling of the pyramidal layer. No-primary sections lacked staining.

Cornu Ammonis 1

The pyramidal cell layer of the CA1 was highlighted with strong punctate perisomatic staining (figure 12). Dendritic-like staining was seen, with projections running from the pyramidal layer into the stratum radiatum (figure 12). Glia were labeled in the stratum oriens and stratum radiatum (figure 12). NRS produced diffuse staining throughout the CA1, with intense labeling of the pyramidale layer. Limited staining was seen in sections incubated with no-primary.

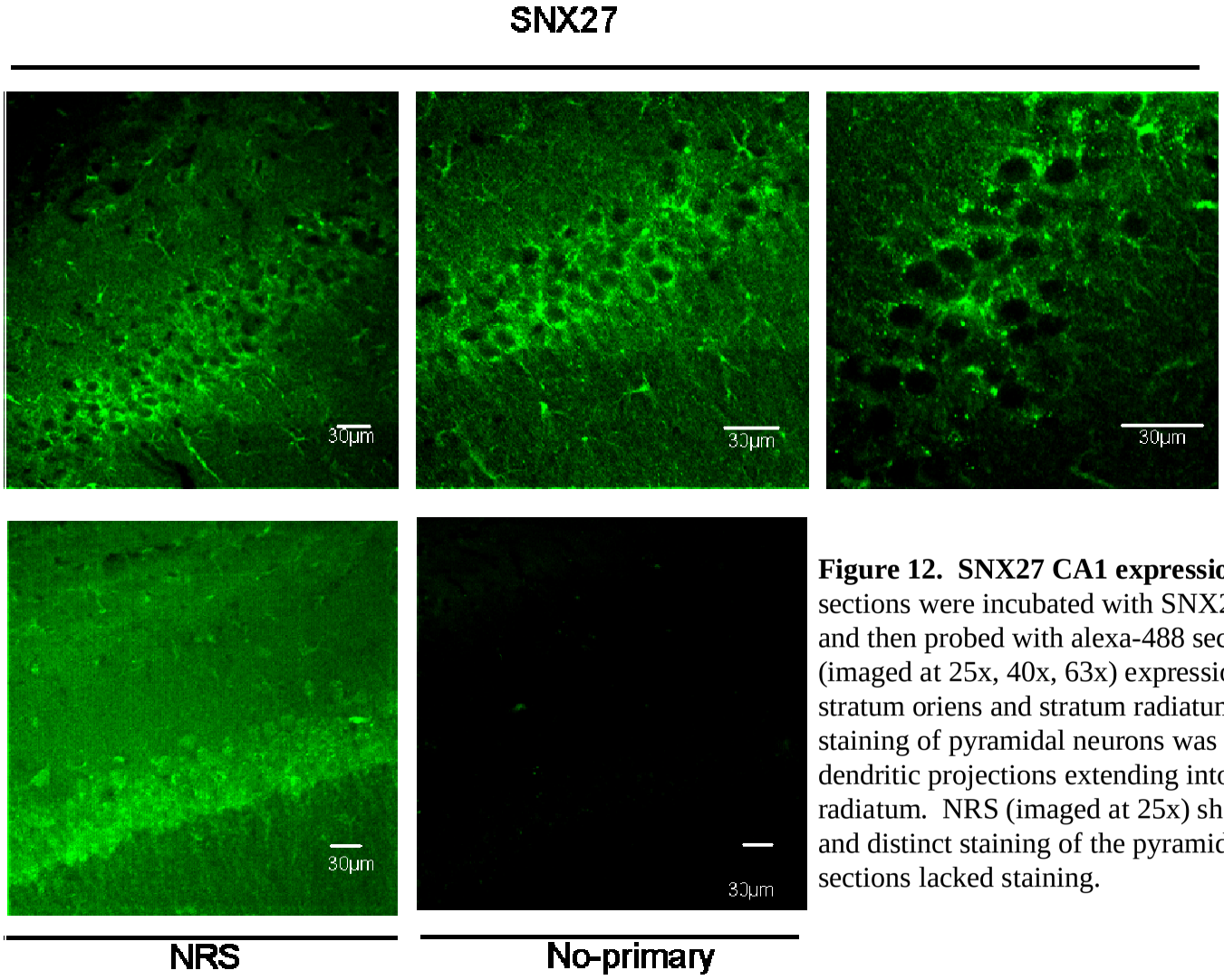

Figure 12. SNX27 CA1 expression. Free floating sections were incubated with SNX27 antibody or NRS and then probed with alexa-488 secondary. SNX27 (imaged at 25x, 40x, 63x) expression was diffuse in stratum oriens and stratum radiatum. Perisomtic staining of pyramidal neurons was punctate with dendritic projections extending into the stratum radiatum. NRS (imaged at 25x) showed diffuse staining and distinct staining of the pyramidal layer. No-primary sections lacked staining.

Discussion:

Switching from DAB to fluorescence allowed for better resolution of the hippocampal layers. The lack of staining in no-primary sections demonstrated that secondary antibodies did not interfere with SNX27 antibody labeling. Although the principle cells of the DG, CA3, CA1 were stained in both NRS and SNX27, the pattern of labeling in these cells was drastically different. NRS produced strong nuclear staining, while SNX27 was perisomatic. There are three plausible explanations for SNX27 perisomatic staining.

Firstly, SNX27 expression could be limited to either dendritic or axonal projections running through the cellular layer, thus outlining the cell bodies of the principle cells. This could account for the projectional staining seen in the DG and CA1. Dendrites of the granule cells extend into the moleculare layer where they synapse with axons from the perforant pathway (O'Keefe et al., 1978). SNX27 expression within these dendrites could lead to the perisomatic and dendritic staining. The DG also contains projections from a variety of interneurons. Depending on the sub-type of interneuron, the soma resides in the polymorph or moleculare layer and sends either axons or dendrites through the cell layer. For example, Chandelier or axo-axonic cells, have dense axons abors in the pyramidale layer (Freund et al., 1996). Similar interneuron innervations occur in the CA1. Thus, staining in the DG and CA1 could be from SNX27 expression in these interneurons. It seems unlikely that staining in the pyramidale layer of the CA3 was the result of coursing processes since only a sub-population of the cells displayed perisomatic staining.

Secondly, SNX27 could be expressed in axons which synapse onto the cell bodies of principle cells. The excitatory synapses formed through the intrinsic enervation of the mossy fibers and Schaffer collaterals form en passage connections to their respective dendrites. These fibers do not enervate directly on the cell body of the principle neurons (O'Keefe et al., 1978). Thus, synaptic perisomatic staining is unlikely to have arisen from these synapses. Inhibitory synapses from interneurons, on the other hand, do directly synapse onto the cell body of principle cells. In particular basket cells traverse the pyramidale and granule layers where their axonal termini end in basket like plexuses around the cell bodies (Frened et al., 1996). This particular connection may account for the polarized perisomatic staining seen in CA3 cells.

Thirdly, SNX27 could be expressed in the cytosol near the plasma membrane. The punctate expression of SNX27 could vesicular in nature. Vesicular expression of SNX27 would be expected given its tendency to target to the early endosome. Thus, it is likely that the punctate perisomatic staining seen in the DG , CA3, and CA1 is localization of SNX27 to the early endosome.

SNX27 expression is likely a combination of these scenarios. Early work in hippocampal dissociated cultures found SNX27 to be expressed in a sub-population of many cell types, both excitatory and inhibitory. Further more, expression was not limited to particular region within these cells, dendritic, axonal, and somal expression was seen (Nassirpour and Slesinger, unpublished).

Although neuronal expression of SNX27 is probable, the clearest labeling with SNX27 antibody was glial. Intense glia staining was consistently found throughout the hippocampus and colocalization studies show a strong correlation between SNX27 and the glial maker GFAP.

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5.3 Determination of Cellular Subtype Expression of SNX27:

Introduction:

My earlier studies of SNX27 expression did not definitively localize SNX27 to a cellular subtype in the hippocampus. To further elucidate SNX27's expression pattern I decided to perform a series of colocalization studies using several neuronal makers. To elucidate the source of the perisomatic staining colocalization between SNX27 and NeuN (neuronal nuclei marker) was investigated. NeuN recognizes the DNA-binding protein NeuN which is very prevalent in the CNS. NeuN strongly labels the nuclei of neurons and faintly stains the cytoplasm. In addition, SNX27 and the dendritic marker MAP-2 (microtubule associated protein-2) were examined for overlapping expression patterns. To explore synaptic expression, SNX27 and PSD-95 colocalization was examined. Lastly, to confirm SNX27 glial expression, colocalization with GFAP (glial fibrillar associated protein) was tested.

Methods:

See previous sections

Results:

Dentate Gyrus

NeuN strongly labeled the cell bodies of the granular layer. Punctate SNX27 staining periscomatically colocalized with NeuN labeled cells (figure 13A). MAP-2 staining presented a diffuse perisomatic pattern in the granular layer with distinct dendritic staining in polymorph layer and more ubiquitous dendritic staining in the moleculare layer (figure 13B). MAP-2 expression overlapped with that of SNX27 in the granular layer, but not the polymorph or moleculare layers (figure 13B). PSD-95 expression was highly abundant, with staining seen nearly throughout the DG, except in the granular cell bodies (figure 13C). The diffuse staining of PDS-95 and SNX27 overlapped (figure 13 C). SNX27 strongly labeled glia which colocalized with the glial staining of GFAP (figure 13 D).

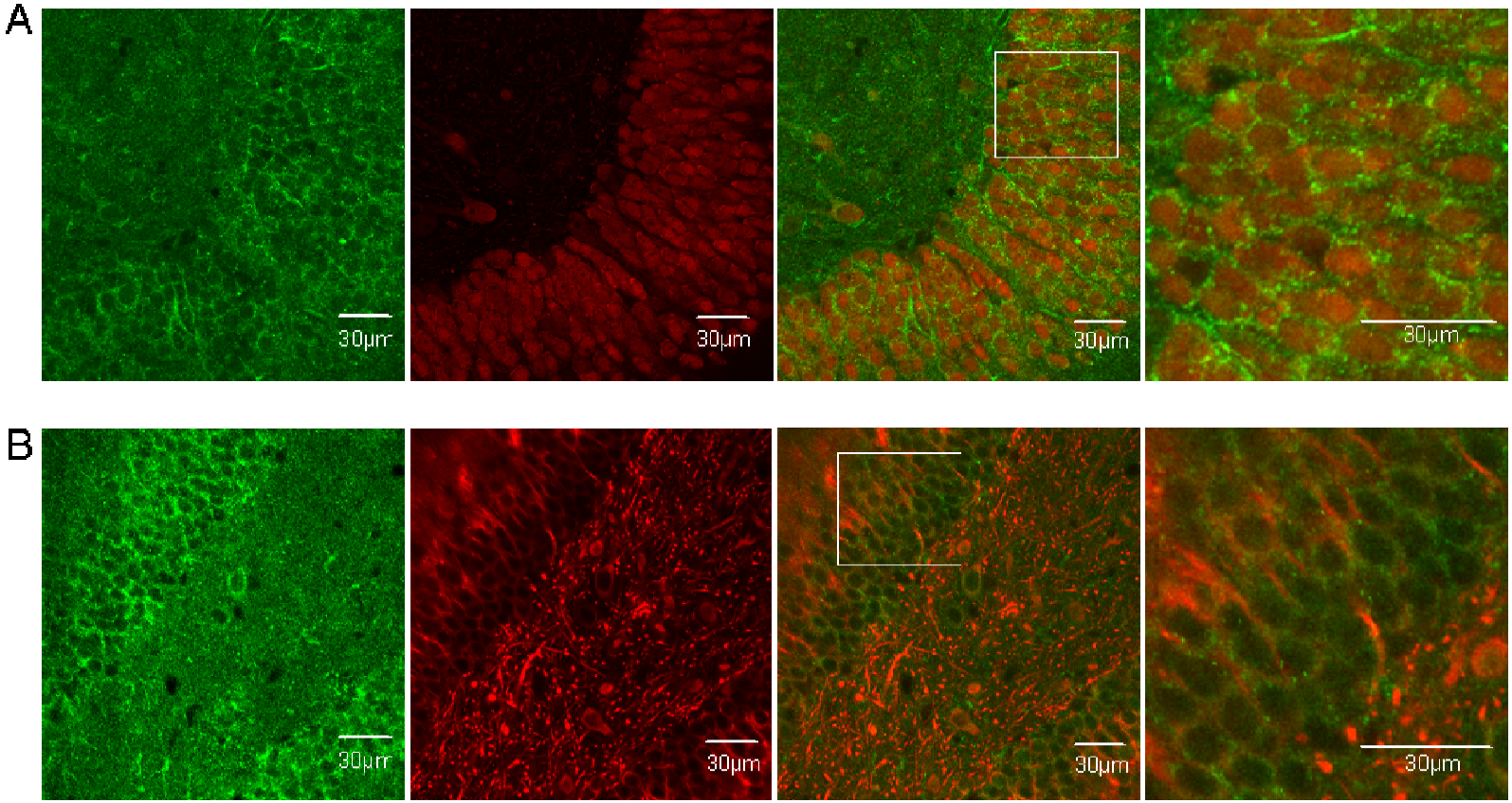

Figure 13. SNX27 DG cellular subtype expression. Free floating coronal sections were co-incubated with SNX27 antibody and NeuN **(A),** MAP-2 **(B),** PSD-95 **(C),** or GFAP**(D).** SNX27 was labeled with alexa-488 (green) and the other antibody with alexa-647 (red). The rightmost images are the merged images of SNX27 and the cell specific marker, with the rightmost of the merged images being a digital zoom of the indicated area. The hippocampus was imaged using laser confocal microscopy (40x)

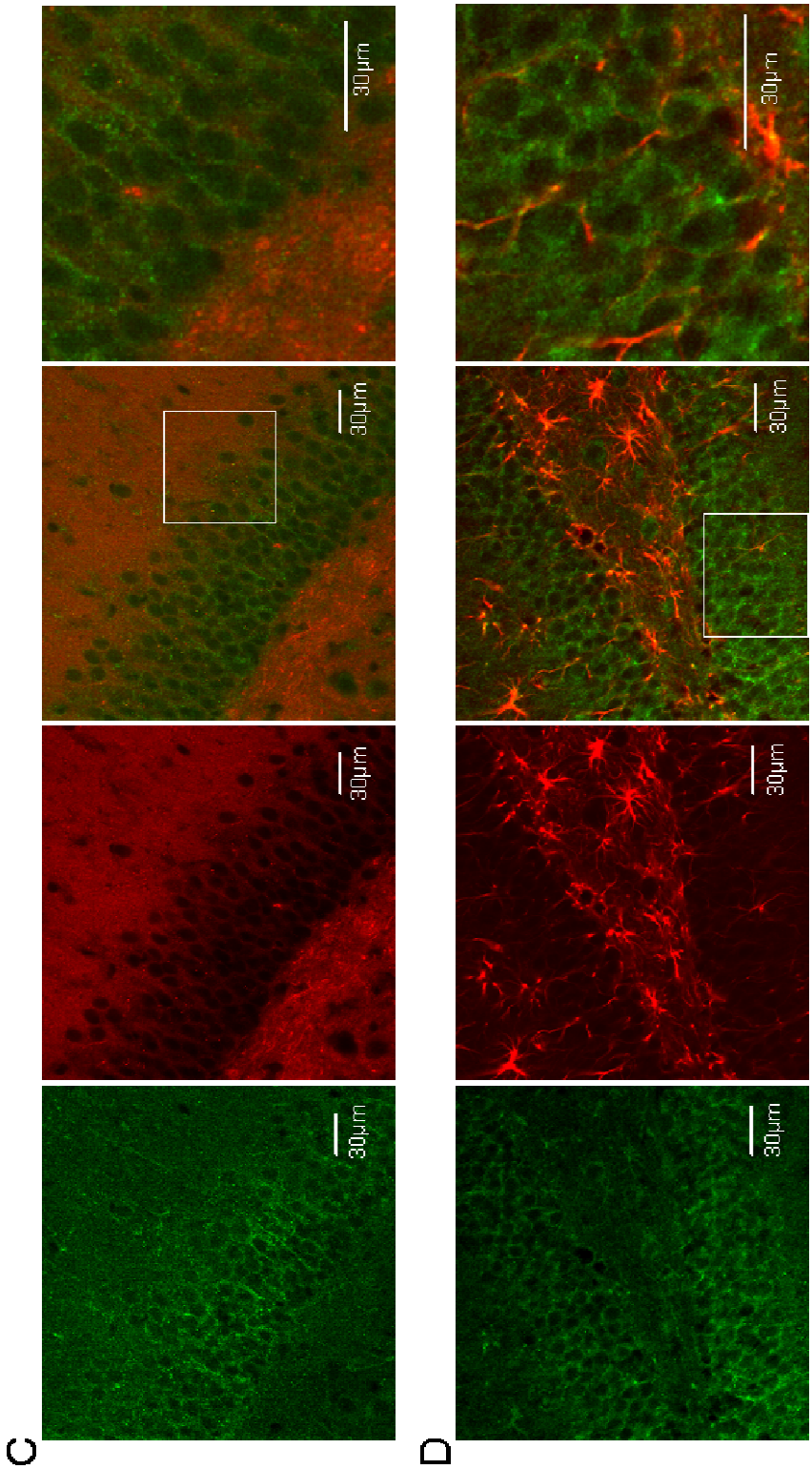

Figure 13. continued

Cornu Ammonis 3

NeuN clearly labeled the soma of pyramidal CA3 neurons. Very few of these cells overlapped with SNX27 staining (figure 14A). MAP-2 distinctly labeled dendritic tracts in both the stratum lucidum and the stratum oriens. SNX27 did not colocalize in these regions. MAP-2 also tracked dendrites running through the pyramidal layer, this staining partially overlapped with SNX27 expression (figure 14B). PSD-95 overlapped with SNX27 expression in the stratum oriens and stratum radiatum with both displaying diffuse staining (figure 14C). GFAP and SNX27 showed a high degree of colocalization in glial cells (figure 14D).

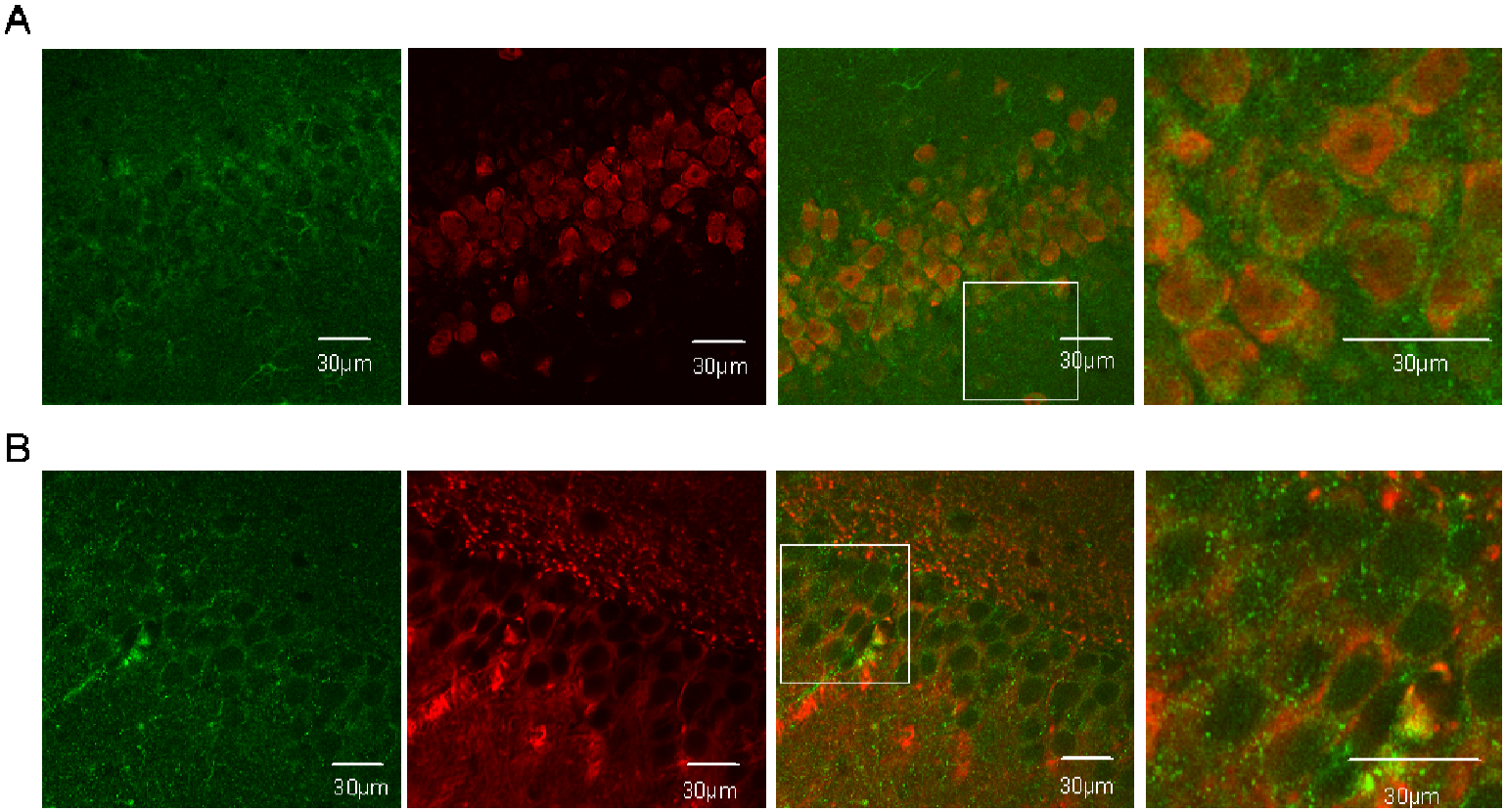

Figure 14 . SNX27 CA3 cellular subtype expression. Free floating coronal sections were co-incubated with SNX27 antibody and NeuN **(A),** MAP-2 **(B),** PSD-95 **(C),** or GFAP**(D).** SNX27 was labeled with alexa-488 (green) and the other antibody with alexa-647 (red). The rightmost images are the merged images of SNX27 and the cell specific marker, with the rightmost of the merged images being a digital zoom of the indicated area. The hippocampus was imaged using laser confocal microscopy (40x)

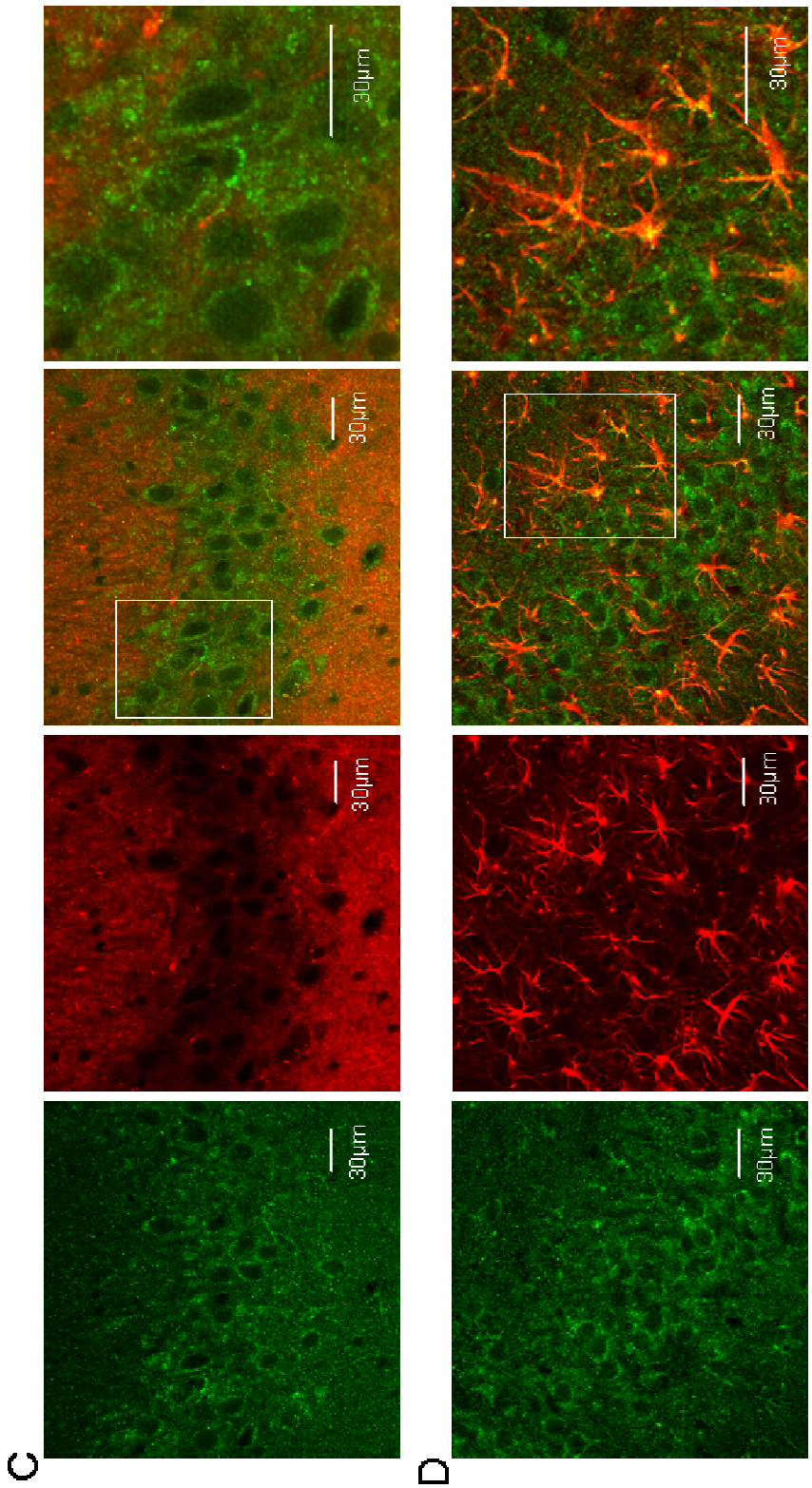

Figure 14. continued

Cornu Ammonis1

Perisomatic SNX27 staining overlapped with NeuN labeled cells (figure 15A). Although both SNX27 and MAP-2 displayed perisomatic staining of the pyramidale layer, SNX27 was much less uniform and only partially overlapped with that of MAP-2. SNX27 expression in the alveus and stratum oriens did not mimic MAP-2 dendritic staining (figure 15B). PSD-95 and SNX27 colocalized in all regions of the CA1; although, PSD-95 displayed some intense projectional staining that was not seen with SNX27 (figure 15C). GFAP colocalized with distinct glial staining in SNX27 (figure 15D).

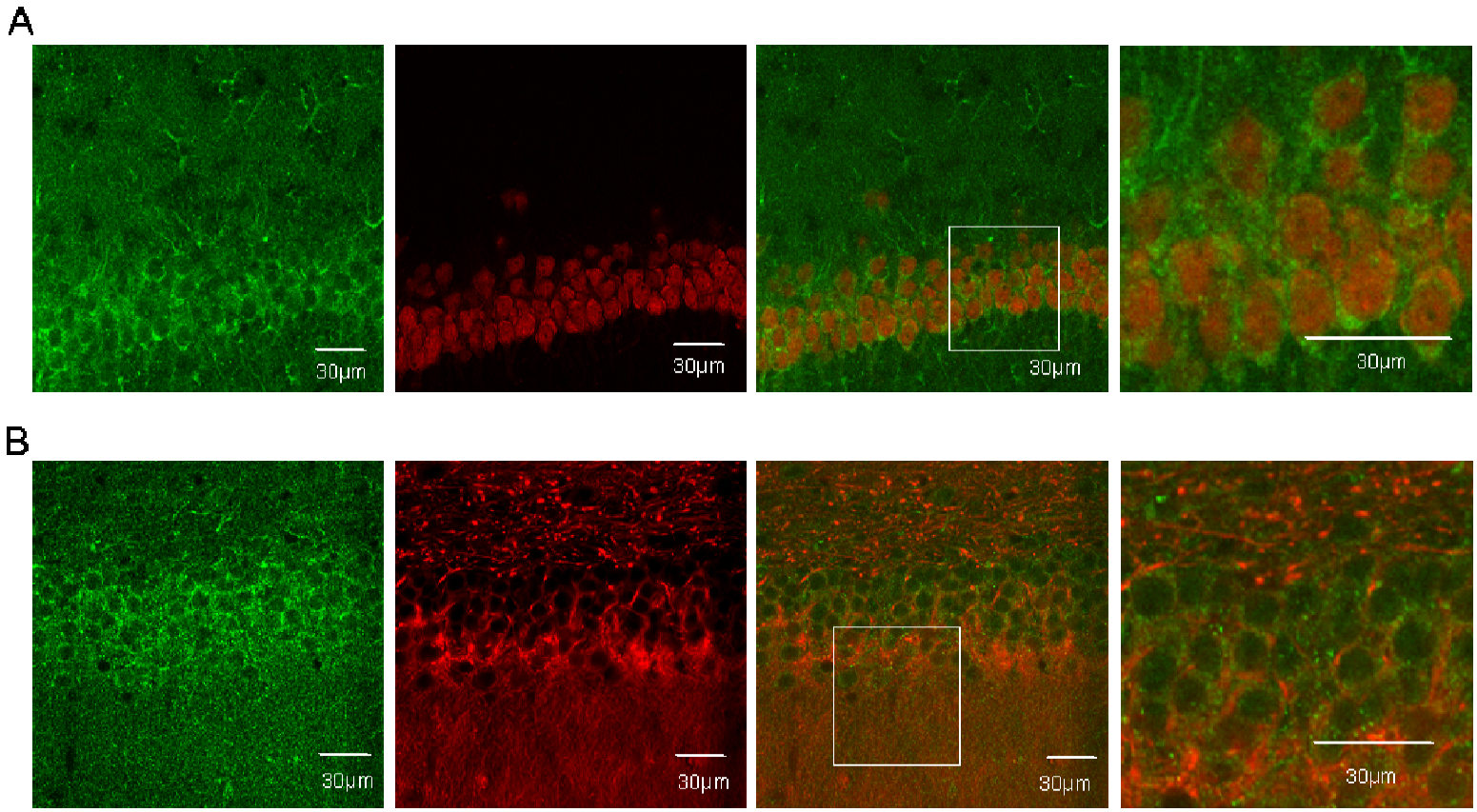

Figure 15. SNX27 CA1 cellular subtype expression. Free floating coronal sections were co-incubated with SNX27 antibody and NeuN **(A),** MAP-2 **(B),** PSD-95 **(C),** or GFAP**(D).** SNX27 was labeled with alexa-488 (green) and the other antibody with alexa-647 (red). The rightmost images are the merged images of SNX27 and the cell specific marker, with the rightmost of the merged images being a digital zoom of the indicated area. The hippocampus was imaged using laser confocal microscopy (40x)

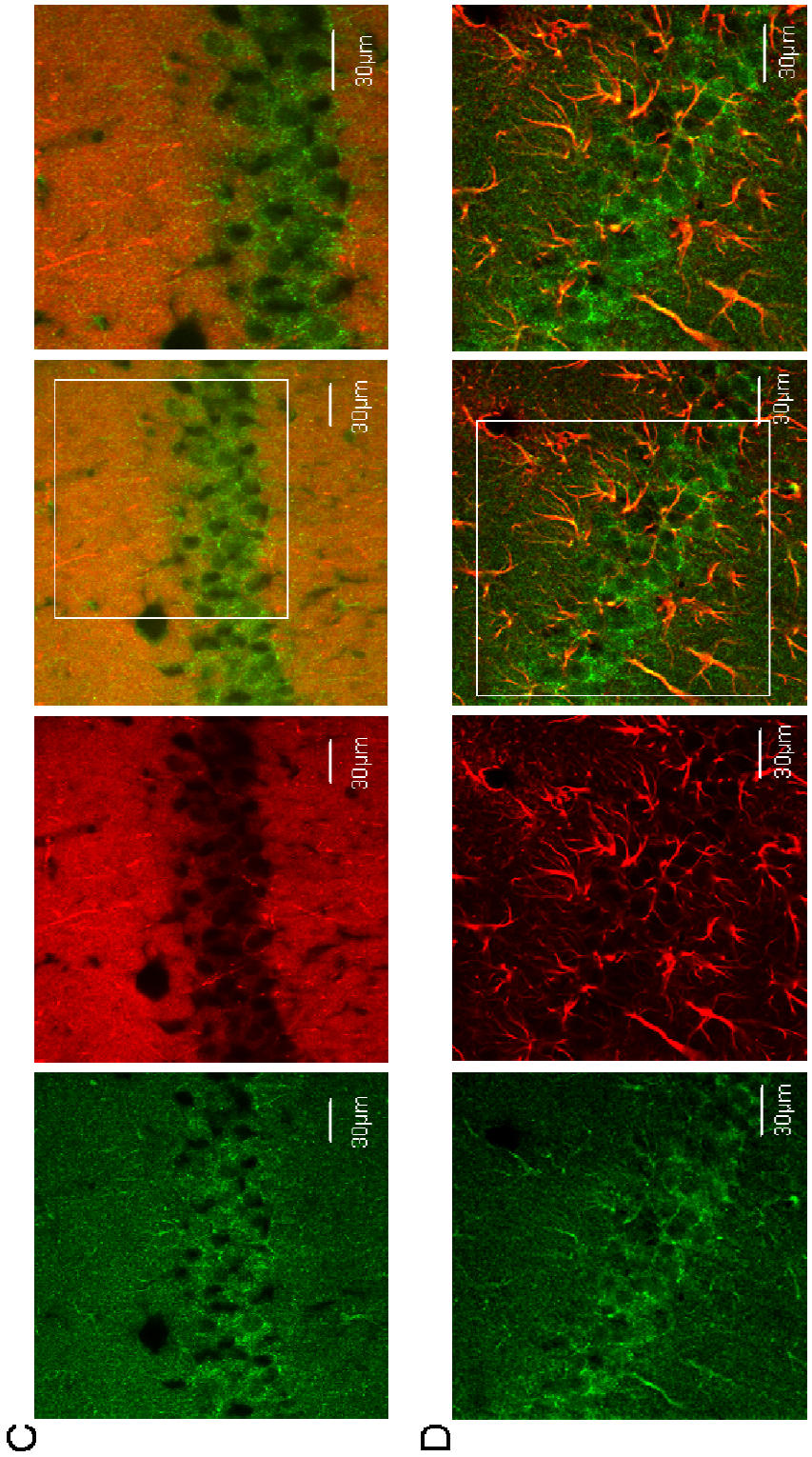

Figure 15. continued

Discussion:

Colocalization studies suggested that SNX27 is expressed in a wide set of cells in the hippocampus. SNX27 is most certainly found in glia, perhaps both microglia and astrocytes. In addition, SNX27 partially colocalized with NeuN and MAP-2 in certain regions of the hippocampus.

To try to distinguish if the punctate perisomatic staining was cytoplasmic or extracellular, such as a dendritic or synaptic, SNX27 was compared to NeuN. However, NeuN preferentially stains the nucleus of the cell and staining strength decreases in the cytoplasm making difficult to define the boundary of the soma. In both the DG and CA1 punctate perisomatic staining seemed to fall within the soma- suggesting a vesicular expression. MAP-2 expression showed a good degree of similarity with SNX27 expression, especially in the granular and pyramidale layers of the DG and CA1 respectively. This overlap supports a postsynaptic function for SNX27. Granule apical dendrites and pyramidal dendrites extend into the moleculare layer and stratum radiatum respectively (O'Keefe, 1978) which are the layers in which projections were seen to extend.

PSD-95 expression was very diffuse, which is likely due to the abundance of the protein (Arnold et al., 1999). This abundance made it difficult to discern specific localization of the protein, severely hindering my ability to compare its expression to SNX27. No convincing conclusions could be drawn from the colocalization of SNX27 and PSD-95.

SNX27 expression in the CA3 was distinctly different from that of the DG and CA1. GFAP colocalization certainly confirmed SNX27 glial expression. NeuN labeling revealed a significantly higher density of pyramidal cells than were labeled with SNX27, suggesting that expression of SNX27 may be limited to a sub-set of CA3 pyramidal cells. The CA3 gives rise to two major excitatory afferent pathways: the Schaffer collateral and bundles that connect to the lateral septal nucleus (O'Keefe, 1978). Thus, SNX27 could differentially be expressed in one of these pathways. SNX27 does not seem to be expressed in the efferent pathways of the CA3. SNX27 expression does not coincide well with that of MAP-2 and no distinct labeling was seen in the stratum lucidum or stratum moleculare where mossy fibers innervate the CA3.

These colocalization studies further the notion that SNX27 is expressed in a variety of cell types throughout the hippocampus. In the DG and CA1 SNX27 seems to most likely glial with some dendritic and vesicular expression. CA3 expression differed from the DG and CA1, with the majority of SNX27 expression being glial and vesicular in a sub-population of CA3 cells.

CHAPTER 6: Determination of GIRK3 Expression

6.1 Determination of GIRK3 Specificity:

Introduction:

To expand on the implications of SNX27 expression I wished to examine its association with GIRK3. Early work had shown that SNX27 and GIRK3 interact through SNX27's PDZ domain. This interaction in turn modulates the trafficking of GIRK3 away from the surface and decreases channel currents (Lunn et al., 2007). If this interaction occurs in native tissue, one would expect both SNX27 and GIRK3 to have similar expression patterns. However the lack of a highly specific GIRK3 antibody has hindered the ability to test this. I acquired a new GIRK3 antibody. The characterization of this antibody was limited; thus, before using the antibody for further studies I wished to confirm its specificity. I tested specificity through western blot analysis.

Additional Methods:

Antibody:

GIRK3: Polyclonal rabbit anti-GIRK3 antibody was graciously supplied by Raphael Lujan.

Results:

GIRK3 antibody detected GIRK3 protein collected from transfected HEK293 cells at the appropriate band size of 44 kDa (figure 16). The antibody also detected a 70 kDa protein from cell lysates collected from HEK293 cells transfected with GIRK3-YFP. No protein was detected in samples collected from untransfected cells (NT).

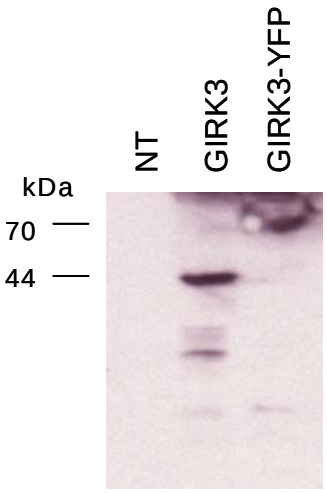

Figure 16. Specificity of GIRK3 antibody. Protein from HEK293 cells transfected with either GIRK3 or GIRK3-YFP were loaded onto a 12% gel for SDS-PAGE, transferred to a nitrocellulose membrane and incubated with anti-GIRK3 rabbit polycolonal antibody. Probing with GIRK3 antibody displayed strong specificity at the appropriate band size and the corresponding shift for GIRK3-YFP. Protein samples from untransfected HEK293 cells lacked antibody recognition.

Discussion:

The GIRK3 antibody detected the appropriate 40 kDa GIRK3 protein. The specificity of this interaction was supported by the antibody's recognition of GIRK3-YFP protein at 70 kDa. Detection for both proteins was in the presence of little background, and protein loaded from untransfected HEK293 cells showed no interaction with the GIRK3 antibody. Taken in the conjunction, these results demonstrate that the antibody was specific.

6.2 Determination of GIRK3 Expression

Introduction:

Studies have shown GIRK3 to be expressed in a variety of hippocampal regions. *In situ* studies have shown GIRK3 mRNA to be expressed in the DG, CA3, and CA1 of rat hippocampus (Karschin et al. 1996). In addition, immunohistological studies have shown protein expression throughout the hippocampus, with especially strong expression in the CA3 and DG hilus (Eulitz et al. 2007). In addition it has been demonstrated that GIRK3 channels are expressed in hippocampal interneurons (Grosse 2003). Having demonstrated specificity of the GIRK3 antibody I wished to characterize GIRK3 expression in the hippocampus.

Methods:

See previous sections

Results:

GIRK3 displayed intense staining in the stratum lucidum of the CA3 as well as the in the stratum moleculare of the CA1 (figure 17A). Punctate staining was seen in the cellular layers of the DG, CA3, and CA1 (figure 17B).

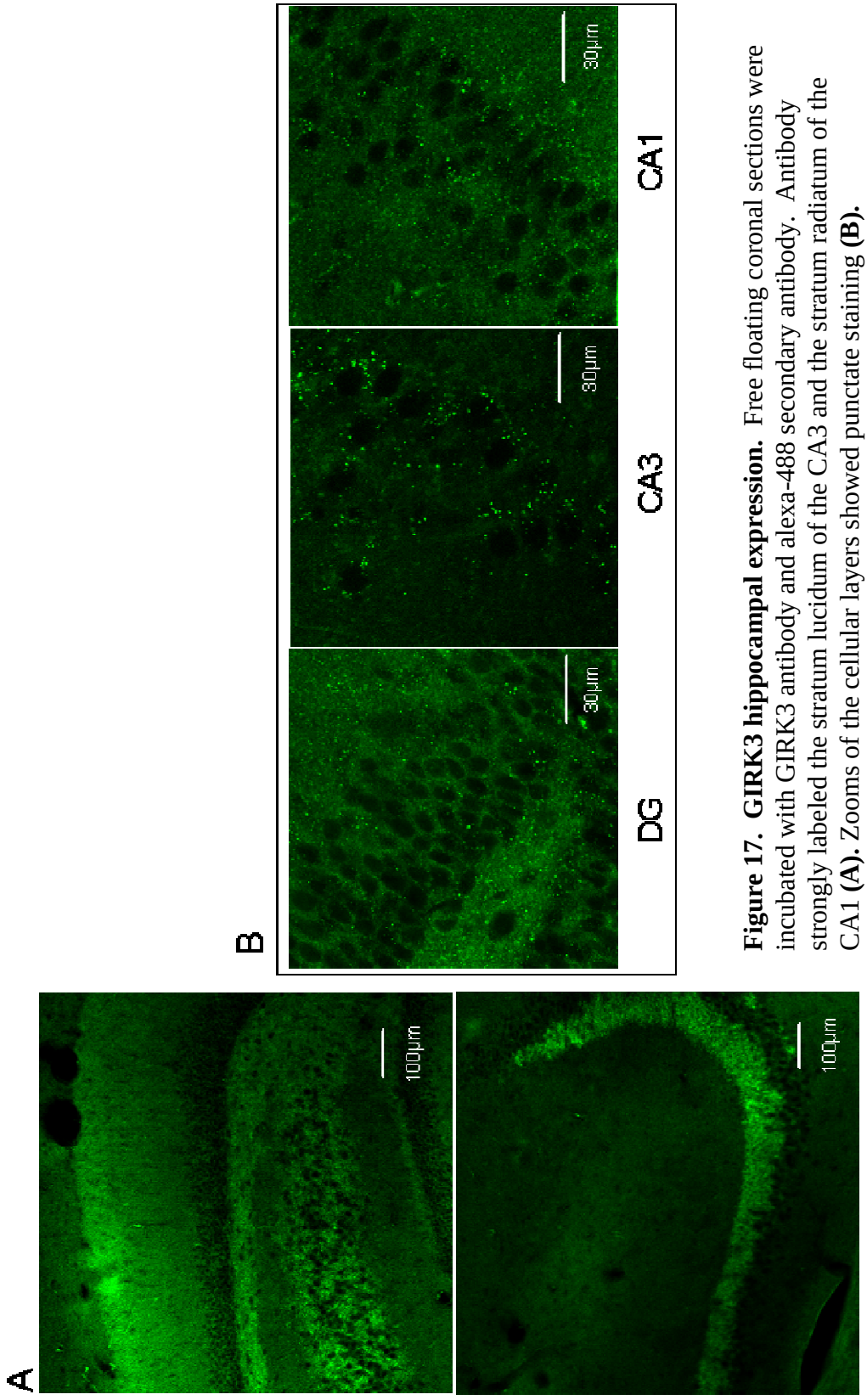

Figure 17. GIRK3 hippocampal expression. Free floating coronal sections were incubated with GIRK3 antibody and alexa-488 secondary antibody. Antibody strongly labeled the stratum lucidum of the CA3 and the stratum radiatum of the CA1 **(A).** Zooms of the cellular layers showed punctate staining **(B).**

Discussion:

The intense labeling of GIRK3 in the stratum lucidum of the CA3 has been demonstrated to be axonal expression in CA3 interneurons. These interneurons project from the stratum oriens through the pyramidal layer to the stratum radiatum (Groose et al., 2003). The staining in the stratum moleculare highlights the region in which CA3 axons synapse onto CA1 dendrites. Therefore it is possible that this staining may be labeling the axons of the Schaffer collateral. The punctate staining in the cellular layers is likely vesicular, perhaps lysosomal, as GIRK3 is targeted to the lysosome through its lysosomal targeting motif (Ma et al., 2002). These puncta were especially prevalent in the CA1, where GIRK3 could also be localizing to the synapses between interneurons and pyramidal cells. Thus, GIRK3 expression seems to be mostly axonal in the CA3 and CA1, with possible vesicular expression in the cellular layers of the hippocampus.

6.3 Determination of Cellular Subtype Expression of GIRK3

Introduction:

GIRK channels are expressed in many regions of the brain and in a variety of cell types (Karschin et al., 1996). They have been seen in both post and presynaptic neurons, in spines and dendrites, and along the plasma membrane (Koyrakh et al., 2005). Thus, GIRK3 may also have a diverse cellular expression pattern. I wished to explore this possibility by performing colocalization studies using NeuN, MAP-2, PSD-95, and GFAP.

Methods:

See previous sections

Results:

GIRK3 punctate staining in the CA3 did not overlap with NeuN; however, puncta in the DG and CA1 perisomatically labeled cells expressing NeuN (figure 18A, figure 19A, figure 20A). GFAP did not colocalize with GIRK3 (figure 18D, figure 19D, figure 19D). MAP-2 expression in the cell layers of the DG, CA3, and CA1 did not align with GIRK3 expression. In the CA3, MAP-2 labeling in the stratum lucidum was distinctively dendritic, while GIRK3 labeling was intense but diffuse in pattern. (figure 18B, figure 19B, figure 20B). PSD-95 showed a general diffuse staining with

processes extending from the CA1 into the stratum radiatum and the stratum oriens. These projections did not colocalize with GIRK3 (figure 18C, figure 19C, figure 20C).

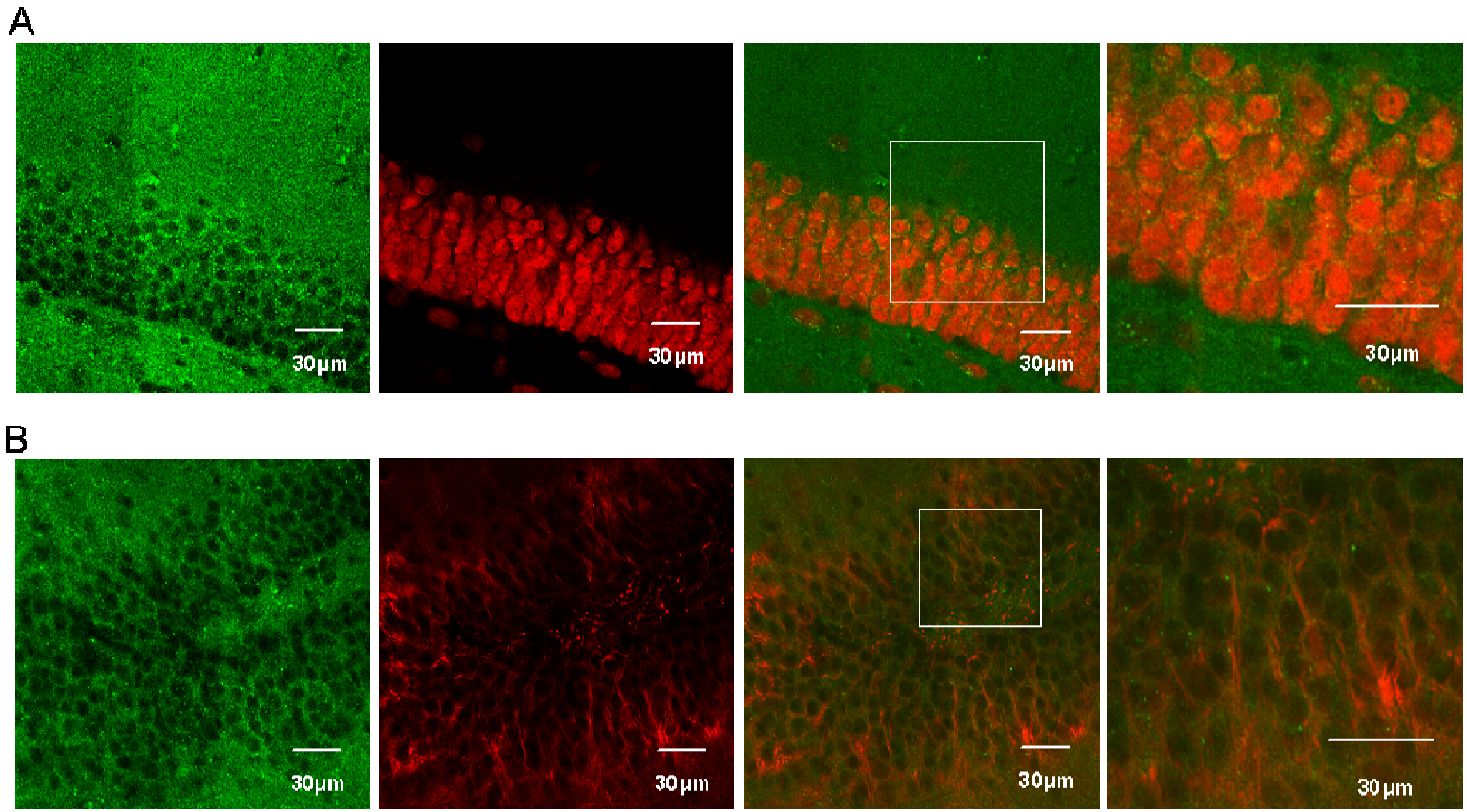

Figure 18. GIRK3 DG cellular subtype expression. Free floating coronal sections were co-incubated with SNX27 antibody and NeuN **(A),** MAP-2 **(B),** PSD-95 **(C),** or GFAP**(D).** SNX27 was labeled with alexa-488 (green) and the other antibody with alexa-647 (red). The rightmost images are the merged images of SNX27 and the cell specific marker, with the rightmost of the merged images being a digital zoom of the indicated area. The hippocampus was imaged using laser confocal microscopy (40x)

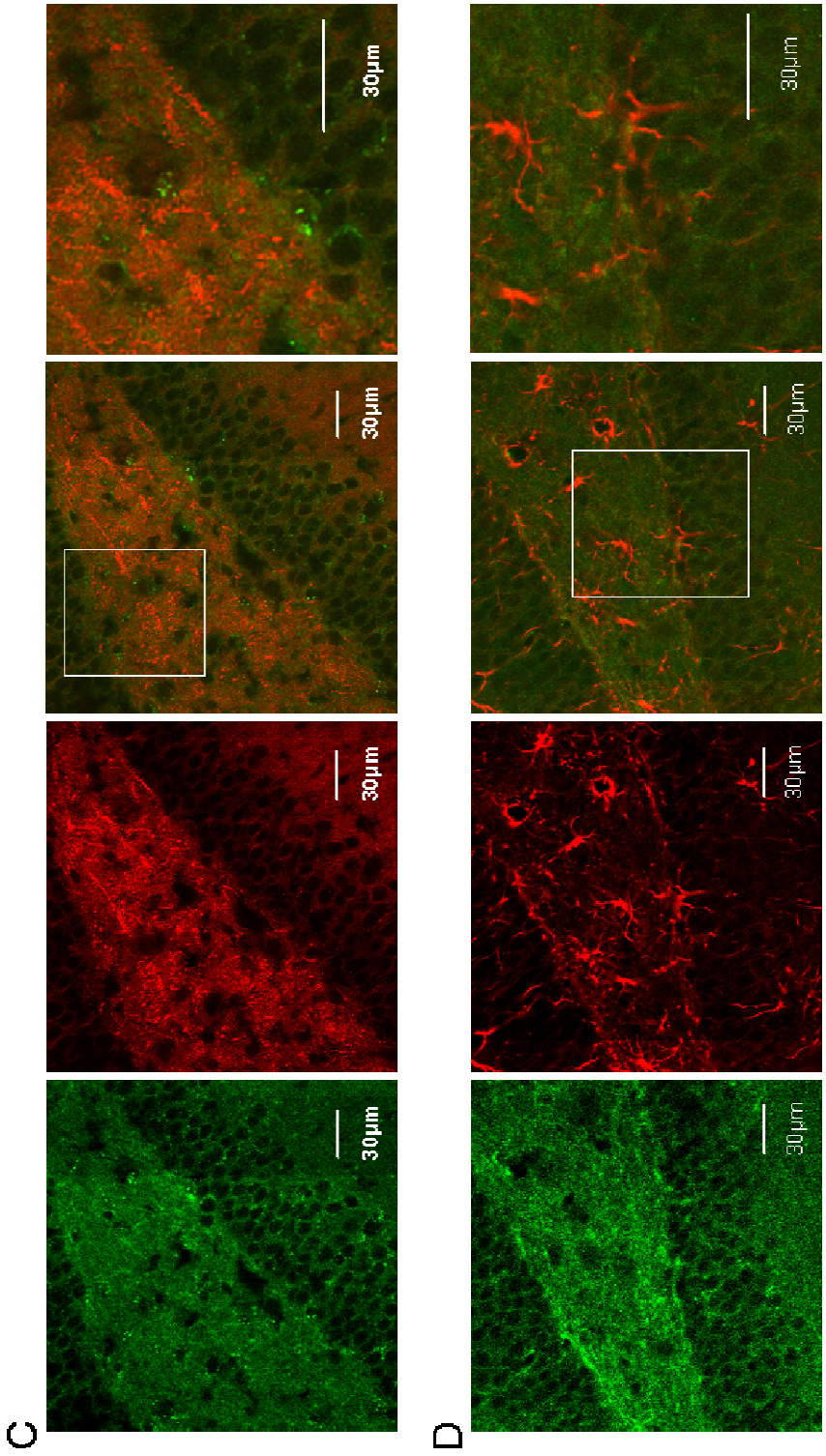

Figure 18. continued

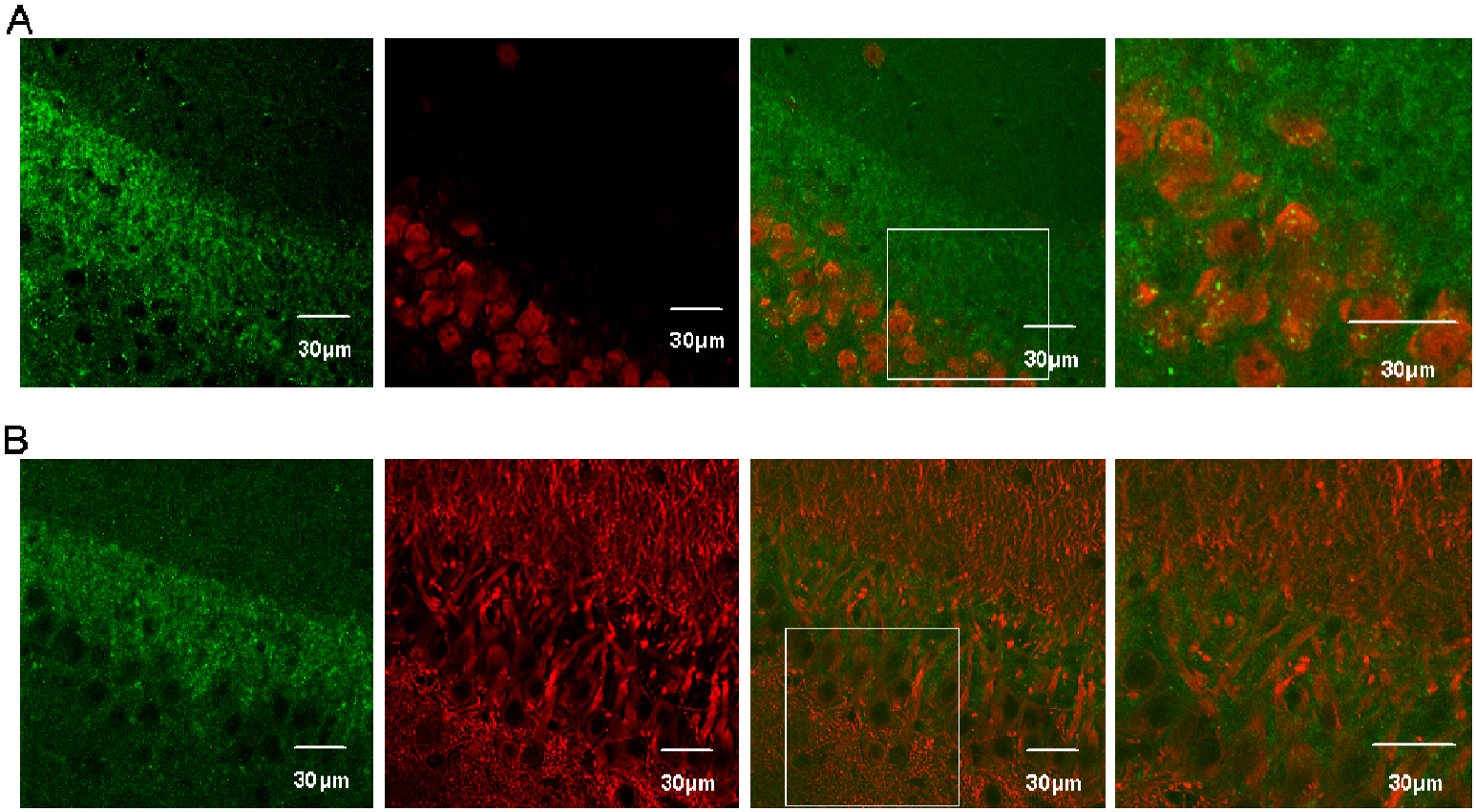

Figure 19. GIRK3 CA3 cellular subtype expression. Free floating coronal sections were co-incubated with SNX27 antibody and NeuN **(A),** MAP-2 **(B),** PSD-95 **(C),** or GFAP**(D).** SNX27 was labeled with alexa-488 (green) and the other antibody with alexa-647 (red). The rightmost images are the merged images of SNX27 and the cell specific marker, with the rightmost of the merged images being a digital zoom of the indicated area. The hippocampus was imaged using laser confocal microscopy (40x)

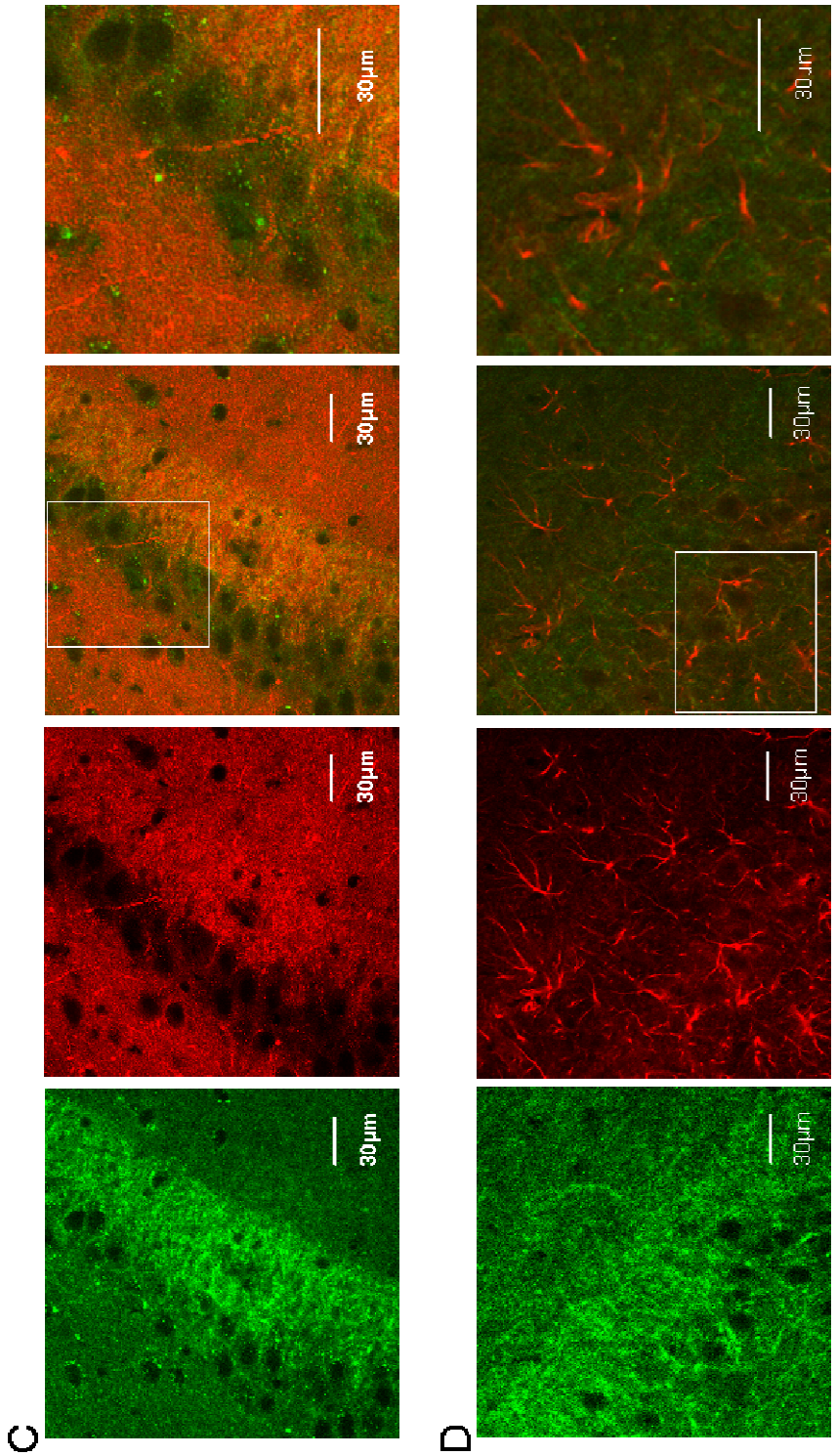

Figure 19. continued

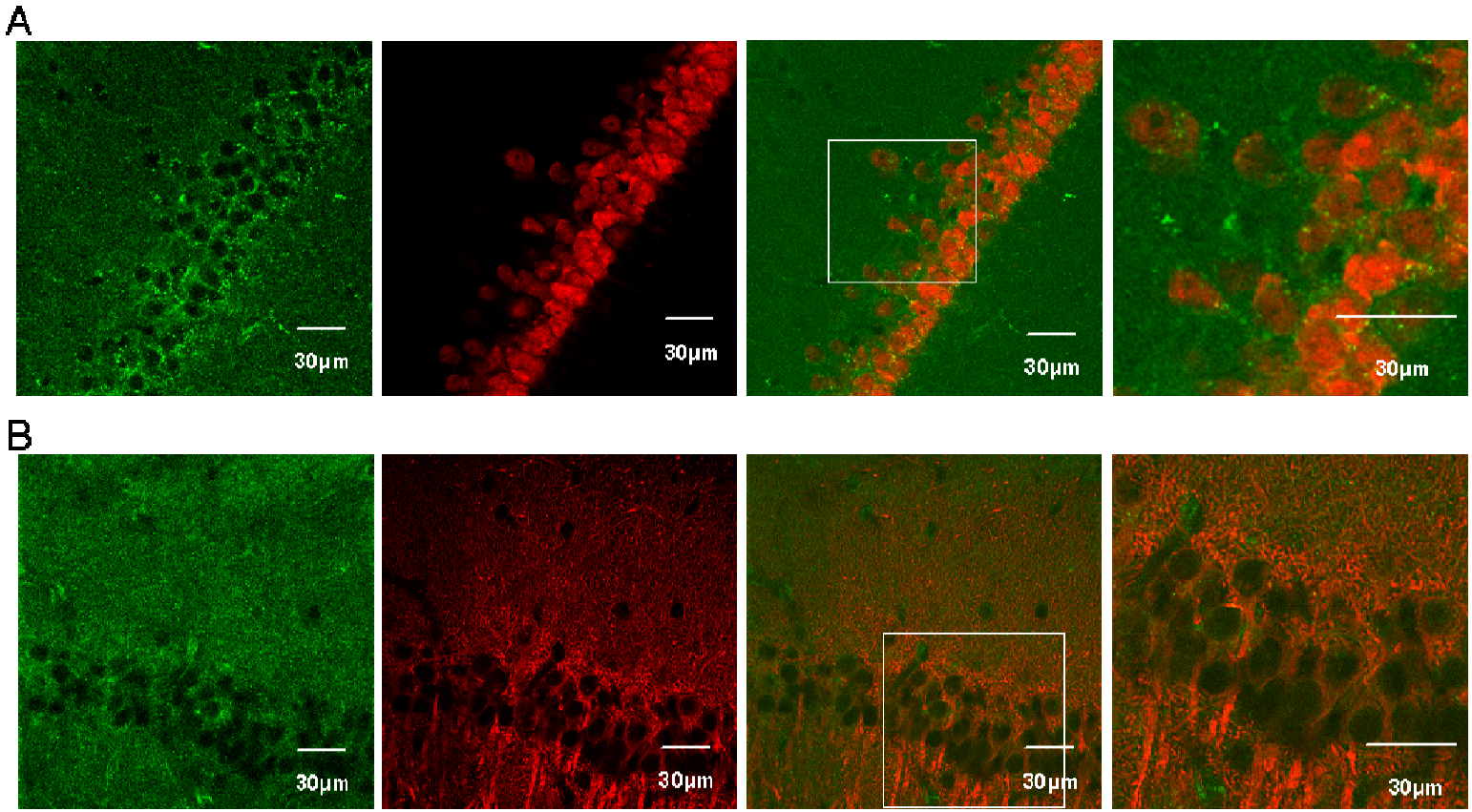

Figure 20. GIRK CA1 cellular subtype expression. Free floating coronal sections were co-incubated with SNX27 antibody and NeuN **(A),** MAP-2 **(B),** PSD-95 **(C),** or GFAP**(D).** SNX27 was labeled with alexa-488 (green) and the other antibody with alexa-647 (red). The rightmost images are the merged images of SNX27 and the cell specific marker, with the rightmost of the merged images being a digital zoom of the indicated area. The hippocampus was imaged using laser confocal microscopy (40x)

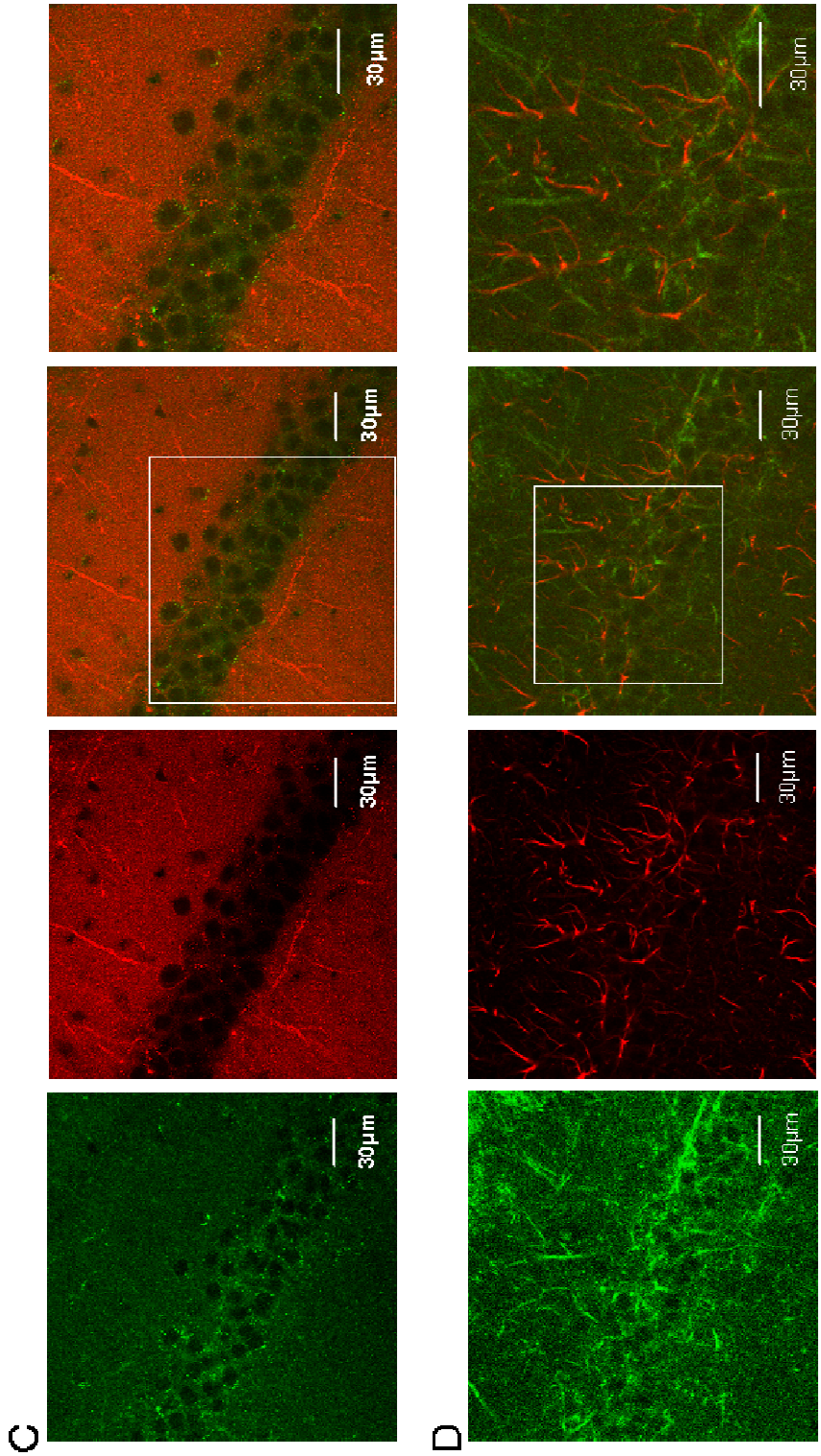

Figure 20. continued

Discussion:

The results of the study suggested that GIRK3 expression is mostly axonal, with some vesicular or synaptic localization in the principle cell bodies. The lack of overlap with MAP-2 in the granular and pyramidale layers suggested that it is not dendritic in these regions. However, dendritic expression of GIRK3 may occur in other regions of the hippocampus as suggested by the intense staining seen in the stratum moleculare. GIRK3 has a lysosomal targeting motif, and studies have localized the GIRK3 subunit to the lysosome (Ma et al., 2002). Thus, the punctate staining may be the result of GIRK3 lysosomal localization. Puncta in the DG and CA1 colocalized perisomatically around NeuN labeled cells suggesting vesicular GIRK3 localization.

6.4 ***Determination of Overlap in SNX27 and GIRK3 Expression:***

Introduction:

Initial *in situ* studies showed overlap of GIRK3 and SNX27 in the DG, CA3, and CA1 (Lunn et al. 2007). Thus, I wished to see if this overlap in mRNA expression correlated to a similar overlap in protein expression. Ideally, to demonstrate this a colocalization study would be performed. However, both SNX27 and GIRK3 antibodies were raised in rabbit, making it impossible to co-incubate them on the same section. However, utilizing the markers used in the earlier colocalization studies as reference points, inferences of SNX27 and GIRK3 overlap could be made.

Discussion:

There appears to be little overlapping SNX27 and GIRK3 expression in the hippocampus. SNX27 expression seems to mostly glial, strongly colocalizing with GFAP. GIRK3 is not expressed in glia. Similarities in GIRK3 and SNX27 colocalization with MAP-2 were very low. Dendritic expression of SNX27 in the DG and CA1 seems likely; however, such a correlation could not be made for GIRK3. Partial overlap with GIRK3 and SNX27 may occur in the punctate staining in the cellular layers. It is possible that the puncta are vesicular, shown by localizing around NeuN labeled cells. Data has shown that these two proteins colocalize in the early

endosome (Lunn et al., 2007). Thus, it is plausible that the puncta seen in both GIRK3 and SNX27 are labeling the early endosome.

Results that suggest poor colocalization between SNX27 and GIRK3 may not be unexpected. The fact that SNX27 expression seems to be modulated by methamphetamine could correlate into a possible drug sensitive interaction between SNX27 and GIRK3. Given the implications of SNX27 and GIRK3 interactions, which results in GIRK3 trafficking to the early endosome and ultimately to the lysosome for degradation (Lunn et al., 2007), it is likely to be tightly regulated. If SNX27 does contribute to drug sensitization through its interaction with GIRK3 you would expect basal interaction and co-expression of these proteins to be minimal but inducible upon drug administration. If this were the case, overlap in expression of these two players may vary depending on drug exposure.

CHAPTER 7: Discussion

The aim of my study was to characterize SNX27 expression in rat brain using a novel SNX27 antibody. First, antibody specificity had to be demonstrated. Western blotting showed the antibody to have good specificity, recognizing a singular band at the appropriate size. With knowledge that the antibody was specific I began to look for gross regional distribution of SNX27 in various brain regions and peripheral organs. As reported in earlier studies (Kaji et al., 2003) SNX27 was found to be expressed throughout the brain and various organs.

Due to its sensitivity to drug sensitization (Kaji et al., 2003) and its implicated role in GIRK3 trafficking I wished to explore SNX27's expression in brain areas implicated in drug addiction. Initial DAB staining showed intense SNX27 expression in the cortex and hippocampus. These locations correlate with earlier data displaying high levels of SNX27 mRNA (Lunn et al., 2007). SNX27 glial expression was seen in both the hippocampus and cortex. SNX27 was also expressed perisomatically in cells throughout the cortex. Expression in the hippocampus was relegated to the cell layers of the DG, CA3, and CA1. However, cell morphology and SNX27 cellular expression in the regions were not discernable using DAB.

Immunoflourescence allowed for better examination of the hippocampus. Hippocampal staining suggested SNX27 expression occurs in a wide variety of regions and cell types. Three main patterns of expression were seen. First, a robust glial expression was seen. It was the most prominent pattern and was seen throughout the DG, CA3, and CA1 in both principle and non-principle cell layers. Second, there was dendritic-like staining through the granular and pyramidal layers of the DG and

CA1 respectively. Some projections were short and could be glial in nature. However, some were longer and extended into the stratum radiatum and moleculare layer. These projections could be either axonal or dendritic. Axonal projections could originate from inhibitory interneurons which project through the principle cell layers. Alternatively, staining could be labeling dendrites from the principle cells. Corroborating this possibility is the overlapping expression seen in the DG and CA1 with MAP-2 dendritic staining. Third, perisomatic staining in the granular and pyramidale layers was seen. This staining was often punctate in nature. This punctate staining could be synaptic or vesicular. Synaptic staining would suggest expression in interneuron that synapse onto the soma of the principle cells. This staining could also result from SNX27 distribution in the early endosomes. SNX27 and GIRK3 did not display much overlap, though they may share expression in the early endosomes in the principle cells. This lack of overlap could be explained as a mechanistic way to prevent sensitization under basal conditions.

I believe SNX27 is a protein expressed in a sub-population of a variety of cells found in the hippocampus. It is expressed in a combination of cells, both glial and neuronal (inhibitory and excitatory). Although SNX27 seems to be ubiquitously expressed a more accurate localization of SNX27 within these cells maybe be achieved if the specificity of the antibody can be improved.

Poor antibody specificity could result from either antibody or methodological inadequacies. Antibody design is crucial for its quality. The SNX27 antibody was generated against the first 19 residues of the N-terminal tail of SNX27. Perhaps this

sequence was not unique enough for the antibody to exclusively detect the protein. Choosing a more unique epitope may increase the antibody's specificity.

Equally, the epitope chosen could be fine but it is inaccessible or destroyed during fixation. Changing the fixation protocol may allow the antibody to access and bind to the antigen more easily. I ran several changes in protocol to try to eliminate background staining. None of these changes increased the sensitivity of the antibody. Background staining could also be the result of sections being too thick, making it difficult to discern SNX27 specific labeling due to cell overlap. Thus, reducing section size may alleviate background staining by making the section more monolayer-like.

Another possibility for high background is the purity of the antibody. It may have a low titre and or a heterogenous mix of SNX27 antibodies of varying affinity. Both of these issues may be alleviated by affinity purification.

Having a SNX27 specific antibody will be an invaluable tool for future studies. So far several binding partners have been identified for SNX27 (Joubert L et al. 2004, MacNeil et al. 2007, Rincón et al. 2007). Due to its unique combination of a PX and PDZ domain and its ubiquitous expression throughout the body, it is likely that SNX27 has other binding partners for which it modulates their trafficking. Identifying these other players may implicate SNX27 in several pathways.

In addition, SNX27's role in sensitization should be further explored. In particular looking at protein expression *in vivo* during methamphetamine sensitization may help expand SNX27's role in addiction. Similarly, looking at modulation in

SNX27 and GIRK3 overlap during sensitization could provide a possible mechanism for induction of sensitization. SNX27 may also interact with the GIRK2c subunit (Lunn et al., 2007). This interaction should be investigated for possible modulation during sensitization as GIRK2c is highly expressed throughout the brain and is the predominate GIRK subunit in the midbrain (Eulitz et al., 2007). If indeed SNX27 activity correlates to drug addiction it may be a possible target for drug therapy. Inhibiting its upregulation or its interaction with GIRKs may block sensitization.

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