GABA SYNTHESIS IN DEVELOPING HIPPOCAMPUS: SNAT1 SURFACES AS A DYNAMIC REGULATOR OF INHIBITORY SYNAPTIC TRANSMISSION

By

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Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in

Pharmacology

May, 2010

Nashville, Tennessee

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To all of the passionate and caring mentors throughout my life,

and

To my family, who bring laughter and joy to my life and are infinitely supportive

ACKNOWLEDGEMENTS

I would first like to acknowledge my mentor, Greg, for his guidance and support throughout my dissertation research. Greg has been intimately involved within nearly every aspect of my doctoral training and I am grateful to him for the extraordinary amount of time he has devoted to me throughout this process. I am also appreciative of his passionate, positive attitude towards science and this dissertation project. His optimism positively influenced my attitude and productivity, both within and outside of the lab. Thank you Greg for your enthusiasm and willingness to be both a friend and mentor, as well as provide an enjoyable learning environment to conduct my graduate studies.

I would also like to acknowledge the remaining members of my committee for their support within and outside of my committee meetings. I would like to thank Dr. Blakely for serving as the chair of my committee and for providing an environment to conduct the synaptosomal uptake experiments.

There are also a number of individuals that provided support for various aspects of my doctoral training, including: Kathryn Lindler, who provided an extraordinary amount of time in the training and troubleshooting of all transport uptake assays; Misty Stafford, who completed the immunohistochemical experiments; Yueli Zhang, who provided western blot training; Dorothy Jones-Davis, who provided a significant amount of preliminary data for this project; Allison Nelson, who troubleshooted specific western blot experiments; Drs. Kevin Haas, Andre Lagrange and Carl Weitlauf, who all provided thoughtful discussions of my research; Karen Gieg and Sandra Camp, who provided

administrative support and patiently answered an endless number of questions and finally, I am grateful for the extraordinary opportunities provided by the past and current leadership within the Department of Pharmacology and BRET office.

I would also like to thank and acknowledge those individuals that are primarily responsible for my completion of this doctoral training program: Dave Engers, my former mentor at Purdue University, is solely responsible for my application to graduate school and provided me with an excellent training environment as an undergraduate. Barbara Thompson and Katie Kelm are both colleagues and friends that have provided persistent guidance, support and immense encouragement during my doctoral studies and during my postdoctoral job search. My fellow graduate students within and outside of Pharmacology have allowed graduate school to be an enjoyable experience filled with laughter. Finally, I would like to thank my family for their support throughout my entire educational training. I am grateful to my parents for the many sacrifices they have made so that I may have the opportunity to complete graduate school. I am grateful to Brandi, Adam and my extended family for the laughter and joy they bring to my life and their encouraging words. Lastly, I would like to thank my husband, Nathaniel, my rock of support. Thank you for bringing a smile to my face and uplifting my spirit every day. Thank you for listening and providing me with daily motivation and encouragement. Thank you for unselfishly and without hesitation moving your life so that I may pursue my dream.

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CHAPTER I

INTRODUCTION

γ-aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the mammalian central nervous system and is synaptically released by local circuit interneurons. In the adult brain, synaptically released GABA activates ligand-gated $GABA_A$ receptors to influx chloride ions, hyperpolarize neuronal membranes and thereby mediate inhibitory synaptic transmission. Because normal brain function requires intact inhibitory synaptic transmission, interneurons must possess mechanisms to adapt synaptic GABA during a wide range of activity states. I hypothesized that one effective mechanism may involve regulating GABA synthesis and vesicular GABA content through modulating the supply of glutamine, an indirect GABAergic metabolic precursor, by System A glutamine transporters. In addition, because GABA serves critical roles during early hippocampal development, I hypothesized that the role of System A transporters at inhibitory synapses also may be developmentlally regulated. Therefore, this dissertation explored if modulation of System A surface activity dynamically regulated GABA synthesis and inhibitory synaptic transmission in response to changing developmental and neuronal activity states.

Therefore, in Chapter I, I will begin with a succinct overview of GABA's functional roles in the context of hippocampal maturation and anatomy, followed by introducing the primary synaptic event measured in studies presented in Chapters II and III. I will then provide a comprehensive review of the known presynaptic mechanisms

that regulate GABA synthesis and vesicular GABA content (the concentration of GABA in a synaptic vesicle). Lastly, I will outline the specific questions driving the hypotheses tested in Chapters II and III.

Maturation and Anatomy of Hippocampus

The hippocampus is located within the temporal lobe of the cerebral cortex and involved in behaviors such as explicit memory, spatial coding and learning as well as in neurological disorders such as temporal lobe epilepsy. Rat hippocampal slices (Figure 1) and synaptosomes from multiple postnatal ages (Postnatal (P) day 7-28) were selected as the model system utilized for all experiments presented in Chapters II and III. Therefore, in this section, I will first introduce the role for neurotransmitter GABA in hippocampal synapse maturation and synaptogenesis. Lastly, I will outline the anatomy of the hippocampus with a focus on neurons located within the CA1 subfield.

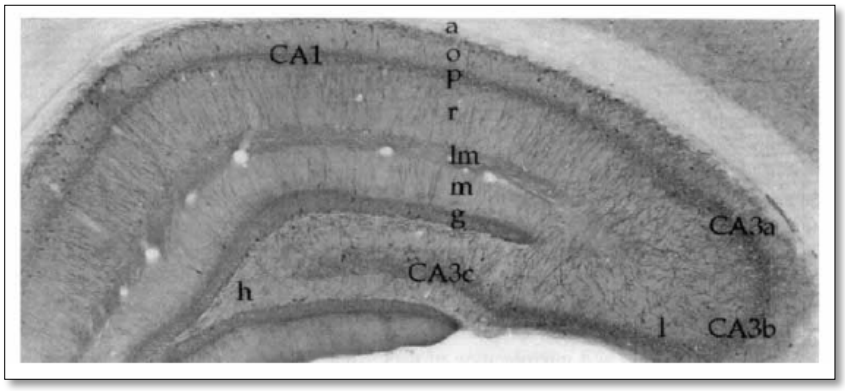

Figure 1. Cellular layers within the hippocampus
A coronal section of rat hippocampus that identifies its multiple cellular layers within the CA1 and CA3 subfields. a, alveus; o, stratum oriens; p, stratum pyramidale; r, stratum radiatum; lm, stratum lacunosum-moleculare; m, stratum moleculare; g, granule cell layer; h, hilus; l, stratum lucidum. Image copied from (Freund & Buzsaki 1996).

Developmental switch in chloride homeostasis

During development, neurons experience a developmental switch in chloride homeostasis. Using intracellular recordings from CA3 pyramidal neurons (the primary principal neuron in hippocampus that releases glutamate, the main excitatory neurotransmitter) in rat hippocampal slices, Ben-Ari and colleagues identified a form of depolarizing neuronal activity termed 'giant depolarizing potentials' (GDPs) in only P0-P8 slices (Ben-Ari *et al.* 1989). The exact postnatal day that GDPs disappear depends upon the brain region and animal species, yet generally occurs prior to the end of the second postnatal week (Ben-Ari *et al.* 2007). GDPs provide the primary source of neuronal activity during early hippocampal development and are characterized by an amplitude of 15-50mV that lasts 300-500ms and a frequency of 0.005-0.2Hz. Activation of $GABA_A$ receptors by GABA underlies the GDPs, as bicuculline (10μM), a $GABA_A$ receptor antagonist, eliminated the depolarizing activity.

In subsequent studies, a reversal in the usual neuronal chloride gradient was found to underlie GABA's depolarizing activity (Figure 2). Immature (P0-P7) pyramidal neurons express the chloride uptake transporter, NKCC1 (named after its electroneutral uptake of 1-Na^+, 1K^+, 2 Cl^- ions) (Marty et al. 2002). NKCC1 accumulates intracellular Cl^- ions and generates an outward concentration gradient for chloride efflux upon activation of $GABA_A$ receptors. Chloride efflux raises the resting membrane potential to chloride's electrochemical potential (E_{GABA}; a more positive potential due to the outward gradient), thereby depolarizing the neuronal membrane. The magnitude of this depolarization is sufficient to remove the voltage-dependent magnesium block from NMDA receptors (Leinekugel *et al.* 1997) and to activate voltage-gated calcium channels

(Leinekugel *et al.* 1995). Consequently, under appropriate conditions, this depolarization can trigger action potentials. Following the first postnatal week, the expression pattern of NKCC1 protein shifts from a somatic to a dendritic localization (Marty et al. 2002). During this shift, pyramidal neurons begin expressing KCC2 (a K^+, $2Cl^-$ co-transporter) mRNA at P5, which gradually increases until P15 where adult levels are maintained (Rivera *et al.* 1999). A similar timeline was identified for KCC2 protein expression by hippocampal pyramidal neurons (Lu *et al.* 1999). KCC2 effluxes intracellular Cl^- ions and generates an inward concentration gradient for chloride influx upon $GABA_A$ receptor activation. Chloride influx decreases the resting membrane potential to E_{GABA} (a more negative potential due to the inward gradient), thereby hyperpolarizing the neuronal membrane. In immature hippocampal neuronal cultures, the triggered influx of calcium upon $GABA_A$ receptor activation induces transcription of KCC2 gene expression, suggesting that GABA itself may mediate the reversal in chloride's concentration gradient (Ganguly *et al.* 2001).

Synapse maturation and synaptogenesis in hippocampus

The timeline for neurogenesis and synapse maturation in rat hippocampus is sequential. Genesis of interneurons (Embryonic day 12-13, E12-13) peaks prior to that of pyramidal neurons (E16-19) (Danglot *et al.* 2006) and interneurons receive the first synaptic inputs. At birth (P0), 95% of interneurons (of all cellular layers in area CA1) (Hennou *et al.* 2002) but only 22% of CA1 pyramidal neurons (Tyzio *et al.* 1999) receive synaptic inputs, as determined by recording spontaneous or evoked post-synaptic currents (sPSCs or ePSCs respectfully) from each neuron type in P0 rat hippocampal slices.

Using this approach, Tyzio and colleagues (1999) identified three populations of CA1 pyramidal neurons at P0: 1) Synaptically silent neurons that express functional $GABA_A$ and NMDA receptors; 2) Neurons that only receive GABAergic inputs yet continue to express functional NMDA receptors; and 3) Neurons that receive GABAergic and glutamatergic inputs. In this population, sPSCs and ePSCs were partially blocked by bicuculline and completely blocked by addition of NMDA and AMPA receptor antagonists. However, in some neurons, sPSCs and ePSCs were mediated only by $GABA_A$ and NMDA receptors; while the converse, sPSCs and ePSCs mediated only by $GABA_A$ and AMPA receptors, was never observed. These findings support that GABAergic synapses form prior to glutamatergic synapses. In addition, active NMDA receptor signaling can occur during a period of time when glutamatergic synapses are AMPA receptor-silent. Previous studies have demonstrated that paired presynaptic activity with postsynaptic depolarization removes the voltage-dependent magnesium block from NMDA receptors, increases influx of extracellular calcium, and thus promotes synaptic AMPA receptor expression (Malenka & Nicoll 1997).

Therefore, Tyzio and colleagues (1999) proposed that in immature neurons, GABA's depolarizing activity provides the postsynaptic depolarization required to activate previously silent AMPA synapses. In 2008, Wang and Kriegstein provided further evidence for this hypothesis. To remove GABA's "depolarizing activity", Wang and Kriegstein performed *in utero* electroporation (at E15) of plasmids coexpressing GFP and shRNA against NKCC1 (Wang & Kriegstein 2008). In cortical pyramidal neurons of P0 brain slices, knocking-down NKCC1 protein expression shifted E_{GABA} to -70mV, a more hyperpolarized potential compared to sham slices (-40mV). This result implies that

activation of $GABA_A$ receptors in *Nkcc1*-shRNA-expressing cells would indeed hyperpolarize, not depolarize, the neuronal membrane. In sham slices, $GABA_A$ receptor-mediated sPSCs were recorded in the first three weeks of postnatal life while AMPA-receptor-mediated sPSCs remained restricted to the second and third postnatal week. In *Nkcc1*-shRNA-knockdown cells, the absence of the GABA-induced depolarization delayed detecting $GABA_A$ receptor-mediated sPSCs until postnatal weeks two and three, while AMPA receptor-mediated sPSCs were completely eliminated. Interestingly, the loss of spontaneous AMPA receptor-mediated PSCs could be rescued by coelectroporation of two plasmids expressing: 1) Nkcc1-shRNA and 2) a NMDA receptor mutant that does not require GABA-induced depolarization for NMDA receptor activation. However, coelectroporation of Nkcc1-shRNA with the wildtype NMDA receptor-expressing plasmid rescued the delayed onset of $GABA_A$ receptor-mediated sPSCs, but not AMPA receptor-mediated sPSCs. Therefore, GABAergic and glutamatergic synapses form via different processes. These findings also support that GABA-induced depolarization is necessary and sufficient to activate previously silent AMPA synapses through a NMDA receptor-dependent mechanism (Figure 2).

GABA's "depolarizing" activity thereby allows interneurons to provide the source and target of the first functional synapses during neuronal maturation. Therefore, in hippocampus, GABA orchestrates inhibitory and excitatory synapse maturation. This role for GABA remains restricted to the first two weeks of postnatal life and thus represents one period of the larger developmental window of synaptogenesis (P0-P28). Once a sufficient density of glutamatergic synapses form, the reversal in chloride's concentration gradient occurs (by P14), and GABA assumes its role as the primary

inhibitory neurotransmitter in order to regulate excitatory neurotransmission. Synaptogenesis continues to occur, yet the initial population of "unsilenced" AMPA synapses now supplies the depolarization required to yield functionally mature glutamatergic synapses (Figure 2).

For hippocampal interneurons, their morphological features progressively mature throughout the period of synaptogenesis. The dendritic length of interneurons progressively increases from P0-P20 where their dendritic arbors adopt morphological features at P20 similar to adult interneurons (Lang & Frotscher 1990). Similarly, extension of their axonal arbors gradually increases during the first three postnatal weeks, although details are only complete for the subtype of interneurons making axo-somatic synapses on pyramidal neurons. For example, one study calculated the staining density for the vesicular inhibitory amino acid transporter (vIAAT or vGAT) from confocal images of rat hippocampus in order to mark newly formed GABAergic terminals. vIAAT density in stratum pyramidale was increased 6-fold at P21 compared with P7 in the CA1 subfield and 4-fold in the CA3 subfield (Marty *et al.* 2002). This suggested that the majority of axo-somatic synapses on pyramidal neurons are established between P7-P21. However, at P0, interneuron synapses on CA1 pyramidal neurons localize to stratum radiatum, demonstrating that formation of axo-dendritic synapses likely precedes that of axo-somatic synapses (Tyzio et al. 1999).

The critical role that GABA serves in inhibitory and excitatory synapse maturation suggests that the presynaptic metabolic processes that govern the synaptic pool of neurotransmitter GABA likely play a critical role in hippocampal maturation. Moreover, some of these metabolic processes may be developmentally regulated to

reflect the changing metabolic demands for GABA as it transitions from an “excitatory” to an inhibitory neurotransmitter during postnatal development.

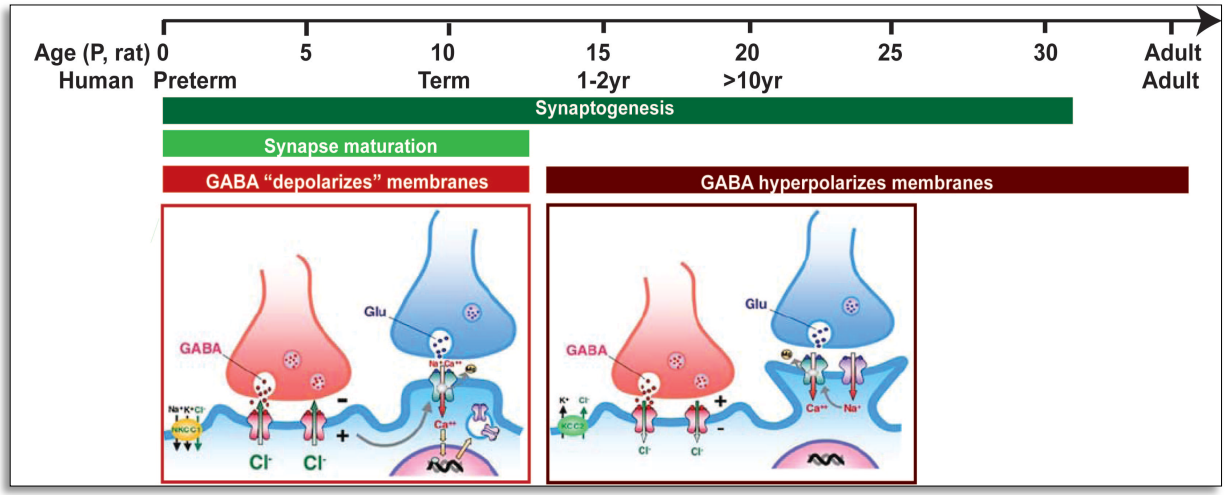

Figure 2. Timeline for maturational processes in rat hippocampus
Developmental timeline in rat postnatal (P) days and its corresponding timeline in humans for three maturational processes in hippocampus: 1) synaptogenesis, 2) synapse maturation and 3) developmental switch in chloride homeostasis reported as the functional consequence of $GABA_A$ receptor activation by GABA (red). The cartoons illustrate the link between GABA's effect on membrane potential with glutamatergic synapse maturation. In immature neurons, GABA's depolarizing activity provides the postsynaptic depolarization required to activate previously silent AMPA synapses. After the reversal in chloride's concentration gradient at P14, the initial population of "unsilenced" AMPA synapses now supplies the depolarization required to yield functionally mature glutamatergic synapses. Cartoons copied from (Wang & Kriegstein 2008).

Circuitry of Hippocampus

Upon maturation, the circuitry of the hippocampus is viewed as a tri-synaptic loop containing three main sub-fields: dentate gyrus, area CA3 and area CA1 (Figure 3). The major input and output of the hippocampus is the entorhinal cortex, which is connected to other regions within the cerebral cortex. Afferent axon collaterals from specific layers within the entorhinal cortex comprise the perforant path and form excitatory synapses on dendrites of granule cells within the stratum moleculare of the dentate gyrus. Granule cells project their axons, mossy fibres, to area CA3, where they form excitatory synapses on dendrites of CA3 pyramidal neurons in stratum lucidum. Axons from CA3 pyramidal neurons comprise the schaffer collaterals, which in turn make excitatory synapses on dendrites of CA1 pyramidal neurons predominantly in stratum radiatum (str. rad.; but also str. oriens) and thus complete the tri-synaptic loop. Lastly, the primary output of the hippocampus consists of axonal projections from CA1 pyramidal neurons that make excitatory synapses on neurons of the entorhinal cortex.

Collectively, five sources (local or extrahippocampal) of glutamatergic afferents innervate distinct cellular layers of the hippocampus (left side, Figure 4). These sources include: 1) schaffer-collaterals, 2) entorhinal cortex, 3) thalamus, 4) amygdala, and 5) local axon collaterals from CA1 pyramidal neurons (Somogyi & Klausberger 2005). Additional inputs include the medial septal area (releasing acetylcholine and GABA) and neuromodulatory fibres releasing norepinephrine, serotonin, or dopamine (Freund & Buzsaki 1996).

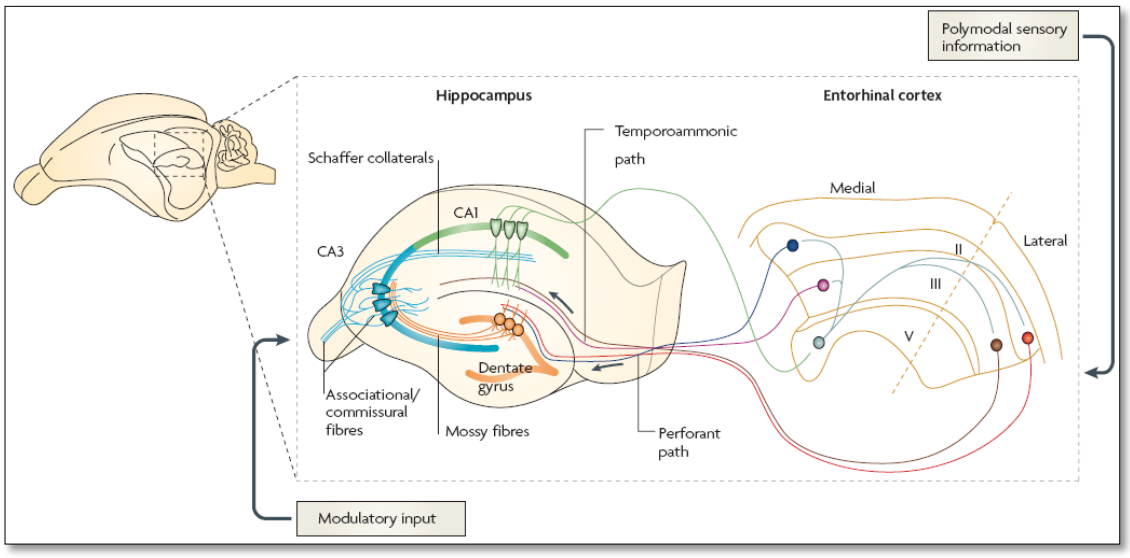

Figure 3. Tri-synaptic circuitry of the hippocampus
Axonal projections within the Perforant, Mossy fibre, and Schaffer collateral pathways comprise the tri-synaptic loop and make excitatory synapses on dendrites of granule cells, CA3 and CA1 pyramidal neurons, respectfully. The entorhinal cortex provides the major afferent inputs to the hippocampus and receives the major efferent outputs from CA1 pyramidal neurons. Image copied from (Neves *et al.* 2008).

Area CA1 pyramidal neurons and interneurons

In hippocampus, pyramidal neurons are relatively uniform in appearance while interneurons comprise a heterogenous population of multiple subtypes. Interneurons diverge in several features, including their dendritic and axonal projections, neuronal firing patterns, and expression of calcium-binding proteins or neuropeptides (Klausberger & Somogyi 2008, Somogyi & Klausberger 2005) (Figure 4). The cell somas of pyramidal neurons lie tightly packed within the pyramidal cell layer and exclusively receive GABAergic inputs from nearby basket cells (Types 2-4, Figure 4). Pyramidal neurons possess two dendritic trees: 1) Apical dendrites that extend into str. rad. and str. lacunosum-moleculare (str. lm.) to receive glutamatergic inputs from schaffer-collaterals, the entorhinal cortex, and the thalamus, and 2) Basal dendrites that extend into str. oriens to receive glutamatergic inputs from schaffer-collaterals, local CA1 axon collaterals, and the amygdala. The majority of axons from CA1 pyramidal neurons project to the entorhinal cortex, but can also extend to the lateral septum, nucleus accumbens, and olfactory bulb.

The localization of an interneuron's somatodendritic compartment and axonal arbor defines the afferent inputs their dendrites receive and efferent outputs their axons target. For example, basket cells express their cell body near the pyramidal cell layer and extend their radial dendrites into str. lm., where they overlay with several distinct glutamatergic inputs. However, their elaborate axonal arbors target the cell body and proximal dendrites of CA1 pyramidal neurons, providing exclusive innervations to this cellular region. Oriens-Lacunosum Moleculare (O-LM) cells (Type 7, Figure 4) localize their cell body in str. oriens where their horizontal dendrites extend, leaving entorhinal

cortical afferents and local CA1 axon collaterals as their main source of glutamatergic inputs. Consistent with their name, O-LM cells project their axons to str. lm., where they target distal apical dendrites of CA1 pyramidal neurons. Interestingly, GABAergic and glutamatergic synapses on distal apical dendrites in str. rad. lie in close proximity to one another, often without intervening astrocytic processes (Lehre & Danbolt 1998). This unique anatomical arrangement facilitates heterosynaptic cross-talk in area CA1 (see 'GABA Synthetic and Metabolic pathways' section for functional implications).

Because interneurons fire action potentials and thus release neurotransmitter GABA at different times and at targeted regions along CA1 pyramidal dendrites, activation of $GABA_A$ receptors provides a diverse spatial-temporal control of pyramidal neuron excitability. As previously noted in the adult brain, activation of $GABA_A$ receptors hyperpolarizes neuronal membranes to shunt excitatory inputs. For a basket cell whose axons solely form perisomatic synapses, this process likely suppresses action potential generation. For O-LM cells that form axo-dendritic synapses, this process likely inhibits dendritic calcium-dependent spikes (Miles *et al.* 1996). Also, whether the interneuron is activated in a feedforward (by the same afferents synapsing on pyramidal neurons, e.g. entorhinal cortex) or in a feedback (by activated pyramidal neurons, e.g. schaffer-collaterals) manner likely influences the functional consequence of $GABA_A$ receptor activation (Spruston 2008). For example, feedforward inhibition shortens the temporal window for excitatory synaptic input integration while feedback inhibition limits continual action potential firing of CA1 pyramidal neurons.

An interneuron's extensive axonal arbor enables it to contact and thus synchronize the excitatory output of hundreds of pyramidal neurons. Hippocampal

pyramidal neurons process cognitive information by firing at distinct frequencies that yield robust oscillatory activity. These frequencies include theta (4-10 Hz) oscillations that occur during exploratory movement, memory tasks, and rapid-eye movement sleep and sharp ripple oscillations (120-200 Hz) that occur during slow-wave sleep and consummatory behaviors (Klausberger & Somogyi 2008). Interestingly, interneurons that fire during these unique frequencies provide the temporal structure required for this oscillatory activity by pyramidal neurons (Klausberger *et al.* 2003). For example, basket cells that fire rhythmically during theta oscillations entrain and synchronize the spontaneous firing of multiple CA1 pyramidal neurons (Cobb *et al.* 1995).

Therefore, interneurons play a dynamic role in neuronal network activity. Their divergent morphological features allow each subtype to provide a unique spatio-temporal control over pyramidal neuron excitability. This critical function places further emphasis on the necessity to understand the presynaptic mechanisms by which interneurons maintain and adapt synaptic GABA during these activity states in both developing and mature hippocampus.

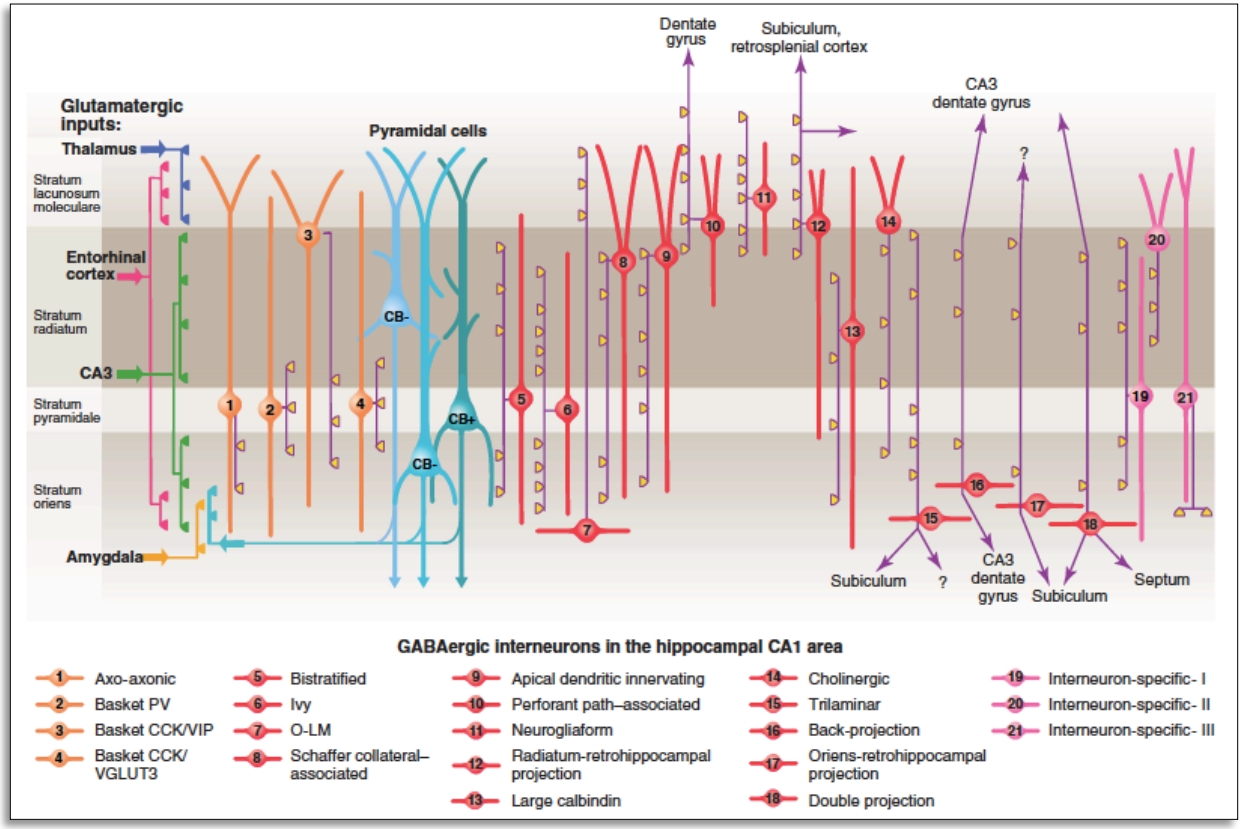

Figure 4. Interneurons expressed in area CA1 of hippocampus
A cartoon illustrating the multiple interneuron subtypes (cell soma and dendrites—orange, red, pink; axons—purple; synaptic boutons—yellow) that innervate specific CA1 pyramidal neuron (blue) domains. Glutamatergic inputs within each cellular layer in area CA1 are labeled on the left. Image copied from (Klausberger & Somogyi 2008).

GABA Receptors and mIPSCs

The previous section identified the multiple roles that GABA plays during hippocampal maturation, including in chloride homeostasis and synapse maturation. In addition, it painted a cellular picture of developing and adult hippocampal neurons, specifically of synapses between area CA1 interneurons and pyramidal neurons. I will now zoom-in to this synapse and provide a brief overview of GABA receptors prior to introducing the remaining components of the GABA neurotransmitter system. Lastly, I will introduce the main synaptic event elicited by these receptors and measured in studies presented in Chapters II and III—miniature Inhibitory Post-Synaptic Currents or mIPSCs (Figure 5).

Ionotropic and Metabotropic GABA receptors

Neurotransmitter GABA mediates fast inhibitory synaptic transmission through phasic activation of postsynaptic ionotropic $GABA_A$ receptors. Upon synaptic release at hippocampal inhibitory synapses, GABA molecules reach a peak cleft concentration near the millimolar range [500μM, (Jones & Westbrook 1995)]. This high concentration enables GABA to synchronously open multiple $GABA_A$ receptors clustered on the postsynaptic cell. The time course of the GABA cleft transient is rapid [100-500μs, (Farrant & Nusser 2005)], partially due to the binding rate of GABA being slower than its diffusion rate (Jones *et al.* 1998). Therefore, synaptic activation of $GABA_A$ receptors likely occurs in nonequilibrium conditions.

$GABA_A$ receptors are members of the cysteine-loop ligand-gated ion channel family and expressed as a heteropentamer that form a chloride and bicarbonate ion-permeable pore. Currently 19 mammalian $GABA_A$ receptors subunits (α1-6, β1-3, γ1-3, δ, ε, θ, π, and ρ1-3) have been cloned, providing a heterogenous and widely diverse array of possible $GABA_A$ receptor combinations. However, the predominant stoichiometry that mediates phasic inhibition contains 2α-2β-1γ ($\alpha_1\beta_{2/3}\gamma_2$, $\alpha_2\beta_{2/3}\gamma_2$, or $\alpha_3\beta_{2/3}\gamma_2$) with a pentamer sequence of γ-β-α-β-α. In this stoichiometry, the α subunit determines the receptor's affinity for GABA [rank order: α6<α1<α2<α4<α5<<α3 (Bohme *et al.* 2004)] and desensitization and deactivation rates [faster kinetics for α1-containing receptors (Farrant & Nusser 2005)]. Moreover, expression of the α-subunit is developmentally regulated, with α3 expression decreasing and α1 expression increasing throughout the first two weeks of postnatal development (Laurie *et al.* 1992). Lastly, $GABA_A$ receptor trafficking, clustering, stability, and endocytosis are all highly regulated processes that contribute to its heterogenous expression and synaptic function (Jacob *et al.* 2008).

In addition to phasic activation of synaptic $GABA_A$ receptors, GABA also elicits tonic activation of extrasynaptic $GABA_A$ receptors. Tonic inhibition is mediated by low (nM - μM range), ambient concentrations of GABA that bind high-affinity extrasynaptic receptors (generally contain 1δ-2$\alpha_{4/6}$-2$\beta_{2/3}$ subunits) (Glykys & Mody 2007). Although the source of this ambient GABA remains controversial, activation of these extrasynaptic receptors provides a tonic input conductance to the postsynaptic cell and may have varying effects on neuronal excitability depending upon neighboring synaptic inputs.

GABA also functions as the primary agonist at both $GABA_B$ and $GABA_C$ receptors. $GABA_B$ receptors are metabotropic receptors coupled to $G_{i/o}$-proteins and

expressed at both pre- and postsynaptic membranes. Upon activation, $GABA_B$ receptors couple their βγ-subunits to potassium channels at postsynaptic membranes to mediate membrane hyperpolarization (Bormann 1988) and to calcium channels at presynaptic membranes to suppress release of vesicular GABA (Couve *et al.* 2000). In addition, presynaptic $GABA_B$ receptors are functional at birth while postsynaptic receptor function is not present until after the first postnatal week (Gaiarsa *et al.* 1995). $GABA_C$ receptors are predominantly expressed in the vertebrate retina and form chloride-permeable ρ-containing homo- or heteropentamers (Bormann & Feigenspan 1995).

Quantal size and mIPSCs

Quantal size is the amplitude of the postsynaptic response to the release of a single vesicle filled with neurotransmitter and is measured as the amplitude of a miniature postsynaptic current (mPSC; see Figure 4 for recording of mIPSCs). Presynaptic and postsynaptic mechanisms exist to control quantal size. If the concentration of transmitter released from a single synaptic vesicle saturates postsynaptic receptors, postsynaptic mechanisms (i.e. receptor number, ligand affinity) determine quantal size. If postsynaptic receptors are not saturated, presynaptic mechanisms (i.e. amount of transmitter released, vesicular transmitter content) determine quantal size.

For interneuron-CA1 pyramidal neuron synapses, synaptic release of a single vesicle filled with GABA does not saturate postsynaptic $GABA_A$ receptors. Therefore, presynaptic mechanisms that modulate GABA's concentration in the synaptic cleft ($[GABA]_{cleft}$) determine quantal size. Through whole-cell recordings of mIPSCs from CA1 pyramidal neurons in acute hippocampal slices, studies have taken two approaches

to support this conclusion: 1) the use of allosteric agonists at $GABA_A$ receptors and 2) direct manipulation of vesicular GABA content. If the amount of GABA in a single vesicle does not saturate $GABA_A$ receptors, allosteric agonists that enhance GABA's affinity for its receptor should augment the chloride conductance, and thus increase mIPSC amplitudes. Indeed, Zolpidem (10μm), an allosteric agonist at $GABA_A$ receptors, increases mIPSC amplitudes (Hajos *et al.* 2000). In addition, enhancing GABA synthesis and vesicle filling augments vesicular GABA content, increases the synaptic $[GABA]_{cleft}$ and thus rapidly increases mIPSC amplitudes (Mathews & Diamond 2003, Hartmann *et al.* 2008). Consistent with changes in synaptic $[GABA]_{cleft}$ reflecting mIPSC amplitudes, reducing vesicular GABA content and thus $[GABA]_{cleft}$ also rapidly decreases mIPSC amplitudes (Zhou *et al.* 2000, Mathews & Diamond 2003). Therefore, at interneuron-CA1 pyramidal neuron synapses, vesicular GABA content is bi-directionally regulated and is a key determinant of the synaptic $[GABA]_{cleft}$ and quantal size.

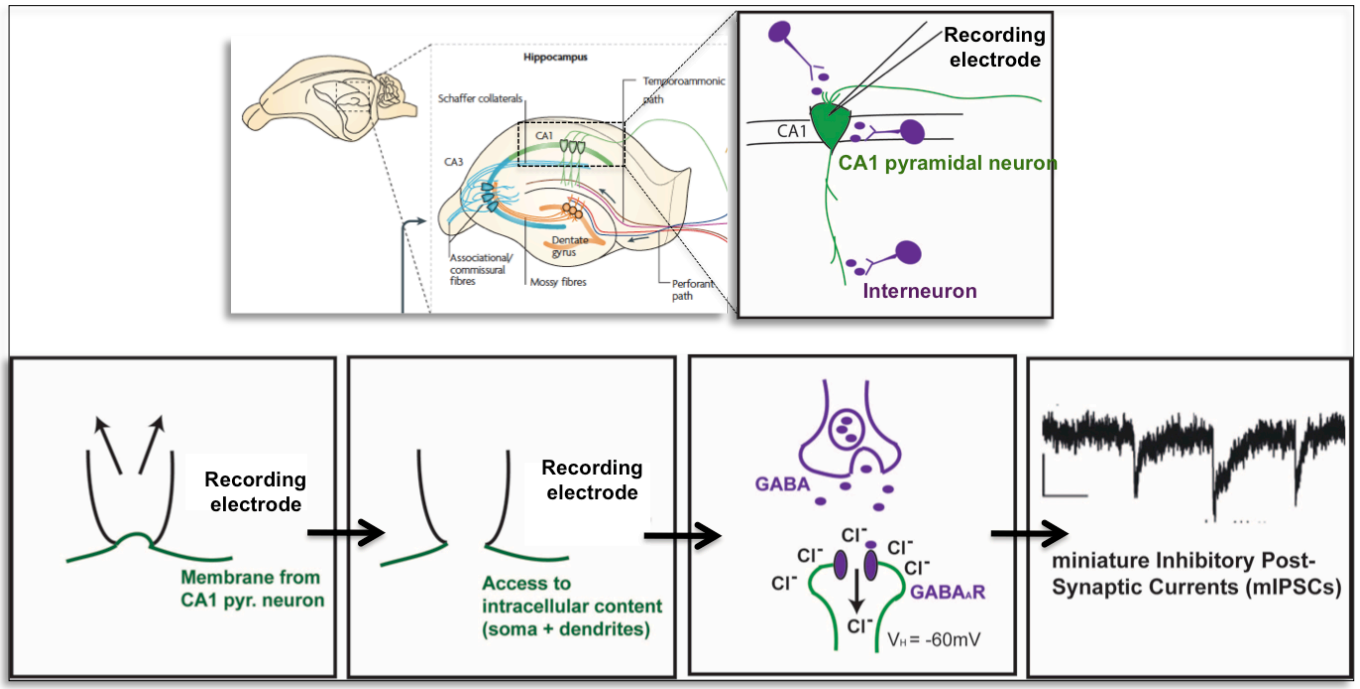

Figure 5. Whole-cell patch-clamp recording of mIPSCs from CA1 pyramidal neurons

Figure 5. Whole-cell patch-clamp recording of mIPSCs from CA1 pyramidal neurons

A step-wise approach to whole-cell recording of mIPSCs from CA1 pyramidal neurons in hippocampal slices using the patch-clamp technique. ***Upper panel (from left to right),*** Area CA1 is outlined in a cartoon of a hippocampal slice. The diagram on the right illustrates a macroscopic view of a cell-attached patch on the cell soma of a CA1 pyramidal neuron (green) using a recording electrode. Interneurons making axo-axonic, axo-somatic, and axo-dendritic synapses are labeled purple (top to bottom). ***Lower panel (from left to right),*** Upon applying a brief suction within the recording electrode (arrows), the cellular membrane is ruptured and the recording electrode now has electronic access to the neuron's intracellular milieu and thus defines the whole-cell recording configuration. Under specific recording conditions, synapses release spontaneous, single GABAergic vesicles in an action potential independent manner. Upon release of a single vesicle, GABA molecules bind and activate influx (or efflux) of chloride ions through ligand-gated $GABA_A$ receptors. Because the neuronal membrane is clamped at a constant holding potential (V_H = -60mV), the current generated from chloride influx at each synapse is integrated at the cell soma and recorded as a mIPSC, the final synaptic output. Scale bar represents 10pA, 20ms.

GABA Synthetic and Metabolic Pathways

In the previous section I described the postsynaptic response by CA1 pyramidal neurons upon release of synaptic GABA by interneurons. Most notably, a single vesicle filled with GABA does not saturate postsynaptic $GABA_A$ receptors at this synapse. As a result, presynaptic processes that govern the synaptic $[GABA]_{cleft}$ provide one mechanism by which interneurons can adapt inhibitory synaptic strength during a range of developmental and activity states. Therefore, in this section, I will provide a comprehensive review of GABA synthetic and metabolic pathways at hippocampal interneuron terminals in an effort to elucidate these presynaptic processes.

Interneurons require ongoing GABA synthesis

In order to maintain synaptic vesicle filling, two primary sources of GABA are available for uptake by the vesicular GABA transporter, vGAT: 1) Pre-existing GABA available from reuptake by GAT-1, the main neuronal GABA reuptake transporter localized at interneuron terminals, and 2) Newly synthesized GABA available from decarboxylation of precursor glutamate by glutamic acid decarboxylase (GAD) (Figure 6). GAD exists as two isoforms, GAD65 and GAD67. GAD65 localizes to synaptic membranes at inhibitory terminals while GAD67 localization is more diffuse, suggesting that GAD65 synthesizes GABA destined for synaptic release (Soghomonian & Martin 1998).

Although reuptake transporters are traditionally viewed as the main source that supplies neurotransmitter for synaptic vesicle filling, multiple lines of evidence indicate

that filling vesicles with GABA does not solely rely on GAT-1 activity. First, in hippocampal (Jensen *et al.* 2003) or neocortical (Bragina *et al.* 2008) slices from GAT1 knock-out mice, neither mIPSC or sIPSC amplitudes are reduced compared to wildtype slices. This suggests that removal of GABA reuptake does not alter vesicular GABA content and thereby the size of the postsynaptic response to single or multiquantal synaptic events. Second, the seizure threshold of GAD65 knock-out mice is reduced compared to wildtype mice (Kash et al. 1997, Asada *et al.* 1996). This finding indicates that loss of synaptic GABA synthesis prevents a compensatory increase in inhibitory neurotransmission during periods of hyperexcitability. Lastly, synaptic vesicles preferentially package newly synthesized GABA over pre-existing GABA. In isolated GAD65-containing synaptic vesicles, vesicular uptake of [^{3}H]GABA converted from substrate [^{3}H]glutamate was actively taken up while uptake of pre-existing [^{14}C]GABA was non-existent (Jin *et al.* 2003). This preferential uptake of newly synthesized GABA is facilitated by GAD65 and vGAT existing as a part of a protein complex on synaptic vesicles (Figure 6). Moreover, this finding demonstrates a functional coupling between GABA synthesis and synaptic vesicle filling (Hsu *et al.* 2000, Jin *et al.* 2003, Wu *et al.* 2007). Taken together, inhibitory synapses require ongoing GABA synthesis to maintain inhibitory neurotransmission.

Developmental expression patterns of GAT-1 and GAD parallel the timeline for synaptogenesis in hippocampus. Although neurons express GAT-1 protein at birth, its uptake activity appears to gain function sometime between P0-P14 (Demarque *et al.* 2002). Positive immunoreactive processes and terminals for GAT-1 gradually increase from P10-P30, where an adult-like pattern of expression is adopted at P30. Interestingly,

GAT-1 expression on interneuron cell bodies transiently increases between P10-P20, but decreases to adult levels by P30 (Yan *et al.* 1997). Like GAT-1, GAD protein expression is present at birth and the number of GAD-positive neurons amongst all cellular layers increases throughout postnatal development and resembles an adult expression pattern by P18 (Seress & Ribak 1988). The correlation between the developmental timeline of GAD protein expression with GABA's developmental roles suggests that the GABA synthetic protein machinery may also contribute to inhibitory and excitatory synapse maturation.

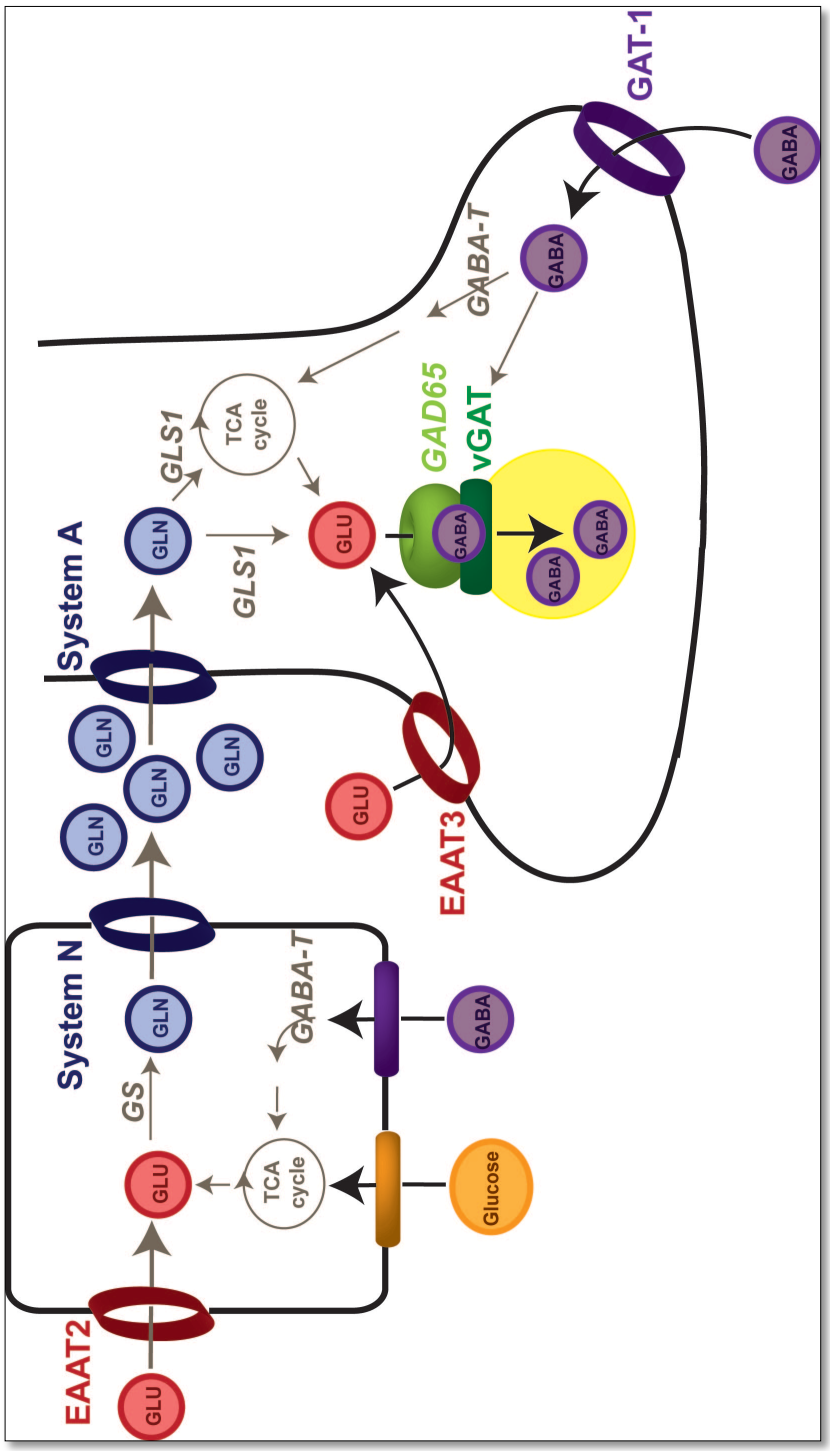

Figure 6. GABA synthetic and metabolic pathways

Figure 6. GABA synthetic and metabolic pathways
A schematic cartoon illustrates a metabolic coupling between astrocytes (left) and inhibitory terminals (right) in order to maintain vesicular GABA synthesis. Transport and enzymatic steps whose contribution to GABA synthesis remains unresolved are illustrated in gray. Two primary sources of GABA are available for vesicular uptake by vGAT: 1) Reuptake of pre-existing GABA by GAT-1 and 2) Newly synthesized GABA through decarboxylation of glutamate by GAD65. GAD65 and vGAT are a part of a complex of proteins that link GABA synthesis with vesicular filling, and thus vGAT preferentially packages newly synthesized GABA over pre-existing GABA. Neurons acquire precursor glutamate primarily through two pathways: 1) Direct uptake of synaptically released glutamate by EAAT3 or 2) Uptake of astrocytic glutamine by neuronal System A transporters followed by conversion to glutamate either directly through the enzyme glutaminase (GLS1) or indirectly through TCA cycle intermediates. Astrocytes express glutamine synthetase (GS), which converts glutamate to glutamine, providing substrate for efflux by System N transporters and for shuttling back to neurons. Astrocytes acquire glutamate through direct uptake by EAAT2 or through multiple enzymatic steps from glucose or GABA catabolism.

Transmembrane transporters supply GABAergic metabolic precursors

In order to maintain ongoing GABA synthesis, inhibitory neurons require a source of glutamate, GABA's immediate metabolic precursor. One intrinsic source relies on neurons synthesizing glutamate *de novo* from glucose through intermediates of the tricarboxylic acid (TCA) cycle. However, neurons do not express pyruvate carboxylase (Yu *et al.* 1983, Shank *et al.* 1985), the necessary anaplerotic enzyme required to replenish the TCA cycle intermediates depleted to maintain *de novo* glutamate synthesis. Therefore, neurons must acquire a supply of glutamate or metabolic precursors of glutamate by transmembrane transporters. In addition, the coupling of GABA synthesis to vesicular GABA filling provides a mechanism whereby supply of these metabolic precursors to inhibitory synapses can dynamically regulate vesicular GABA content and thus inhibitory neurotransmission.

Multiple lines of evidence demonstrate that the Excitatory Amino Acid Transporter (EAAT) 3 is one transmembrane transporter that directly supplies inhibitory terminals with glutamate for vesicular GABA synthesis (Figure 6). First, non-pyramidal neurons in str. rad. and str. oriens in adult hippocampus express EAAT3 mRNA (Berger & Hediger 1998). EAAT3 immunoreactivity localizes to presynaptic terminals in area CA1 of hippocampus (He *et al.* 2000) and co-localizes with GAD in non-pyramidal neurons of cerebral cortex (Conti *et al.* 1998). Second, intraventricular infusion of adult rats with antisense oligonucleotides to EAAT3 reduces newly synthesized [^{14}C]GABA from [^{14}C]glutamate, leading to a reduction in mIPSC amplitudes recorded from hippocampal CA1 pyramidal neurons compared to control rats (Sepkuty et al. 2002).

These deficits in vesicular GABA synthesis lead to hyperexcitability and development of seizures in EAAT3 antisense-treated rats.

Lastly, our lab has demonstrated that supply of exogenous glutamate (Mathews & Diamond 2003) or synaptically released glutamate (Stafford *et al.* 2009) increases vesicular GABA synthesis and thus mIPSC amplitudes in hippocampal slices. Interestingly, the mechanism underlying the latter finding required glutamate spillover from neighboring glutamatergic synapses. As a result, spillover only occurred within inhibitory terminals that make axo-dendritic synapses on CA1 pyramidal neurons, as pyramidal neuron cell bodies are devoid of glutamatergic inputs. The functional consequences of these unique anatomical features within area CA1 are two-fold: 1) Astrocytes express EAAT2 which uptakes the majority of synaptically released glutamate in order to maintain a low extracellular concentration of glutamate (Danbolt 2001). Therefore, EAAT3-mediated glutamate uptake at axo-dendritic inhibitory synapses likely constitutes a feedback mechanism to increase inhibitory synaptic transmission only during periods of excessive glutamate release and thus "spillover". 2) The unique spatio-temporal contribution of this pathway suggests that inhibitory terminals likely rely on an additional GABA biosynthetic pathway to support constitutive vesicular GABA synthesis.

Astrocytic glutamine contributes to ongoing GABA synthesis

In addition to direct glutamate uptake, interneurons can also synthesize glutamate from glutamine. Although interneurons cannot synthesize glutamine, glutamine exists at high extracellular concentrations in rat hippocampus (0.2mM) (Lerma *et al.* 1986) and in

human cerebrospinal fluid (0.5-1mM) (McGale *et al.* 1977). Therefore in contrast to glutamate, glutamine is ubiquitously available to inhibitory synapses for interneuronal uptake. Neurons express phosphate-activated glutaminase type 1 (GLS1) in a cell-type dependent manner (Laake *et al.* 1999), where it localizes to mitochondria and exists in both soluble and membrane-bound forms. GLS1 is an allosteric enzyme regulated by multiple cellular signals that include activation by phosphate ions and feedback inhibition by glutamate (Kvamme *et al.* 2001). GLS1 may synthesize glutamate directly through deamidation of glutamine or indirectly through initial conversion of glutamine to TCA cycle intermediates (Waagepetersen *et al.* 1999) (Figure 6). However, GLS1 expression by hippocampal interneurons has not been shown and cortical cultures from GLS1 knock-out mice reveal impairments in excitatory but not inhibitory synaptic transmission (Masson *et al.* 2006). In addition, a recent study detected glutaminase enzyme activity but not immunoreactivity using current antibodies in cultured astrocytes (Kvamme *et al.* 2008). Therefore, astrocytes and possibly interneurons may express unknown glutaminase isozymes that indeed synthesize glutamate from glutamine at inhibitory terminals. Consistent with a role for glutamine in GABA synthesis, modulating glutamine levels increase (Battaglioli & Martin 1990, Battaglioli & Martin 1996) or decrease (Kapetanovic *et al.* 1993) newly synthesized GABA levels measured by HPLC and GC/MS.

If glutamine indeed exists as a metabolic precursor of neuronal glutamate and thus GABA, what is the source of neuronal glutamine? Van Den Berg and Garfinkel (1971) proposed the first evidence that glutamate and glutamine exist in distinct metabolic compartments—astrocytes represent a small compartment that synthesize glutamine and

neurons represent a large compartment that utilize glutamine to synthesize glutamate. Given that astrocytes selectively express glutamine synthetase (GS) (Norenberg & Martinez-Hernandez 1979), which synthesizes glutamine from glutamate, the existence of a glutamate-glutamine-GABA (glu-gln-GABA) pathway emerged (Benjamin & Quastel 1975). In this pathway, astrocytes synthesize glutamine from glutamate and export glutamine for neuronal uptake, allowing subsequent synthesis of glutamate and GABA in inhibitory terminals (Figure 6). Intrastriatal injections of 10mM MSO, an inhibitor of GS, into the neostriatum of adult rats reduced glutamine and subsequently GABA levels (Paulsen *et al.* 1988). MSO also reduced evoked release of GABA in neostriatal tissue slices (Paulsen & Fonnum 1989).

Despite agreement that astrocytes are the source of neuronal glutamine, the source of glutamate to synthesize astrocytic glutamine remains controversial. One source relies on synthesis of glutamate through TCA cycle intermediates supplied by glucose or GABA catabolism in astrocytes (Figure 6). In co-cultures of cerebral cortical neurons and astrocytes, 10mM MSO reduced neuronal labeling of GABA from [^{13}C]-acetate using [^{13}C]NMR spectroscopy (Sonnewald *et al.* 1993). In adult rats, intravenous infusion of [^{13}C]-glucose enriched neuronal labeling of glutamine and GABA (Patel *et al.* 2001). Adult astrocytes express both GAT-1 and GAT-3 (Ribak *et al.* 1996) and GABA transaminase (GABA-T) activity (Schousboe *et al.* 1983). Upon catabolism of synaptically released GABA, GABA-T synthesizes substrates that feed into the TCA cycle, yet the functional contribution of this source remains unknown. In contrast, 1.4mM MSO in hippocampal slice cultures causes astrocytes to accumulate, but neuronal terminals to deplete glutamate, as visualized by immunogold labeling of glutamate using

electron microscopy (Laake *et al.* 1995). Because astrocytes accumulate glutamate, Laake and colleagues concluded that astrocytic uptake of glutamate by EAAT2, not glutamate synthesis through the TCA cycle, is the source for synthesis of astrocytic glutamine. Further experiments examining the role of EAAT2 in GABA synthesis will be required to distinguish which source of glutamate serves to synthesize glutamine for participation in the glu-gln-GABA pathway.

System A Sodium-coupled Neutral Amino acid Transporters (SNATs)

The previous section presented evidence that an EAAT3-independent GABA biosynthetic pathway exists and likely involves supply of astrocytic glutamine. Therefore, in this section, I will present current evidence supporting and opposing a role for neuronal System A Sodium-coupled Neutral Amino Acid Transporters (SNATs) as the suppliers of astrocytic glutamine to interneurons. Also, I will discuss their protein expression pattern, their known regulators of transport activity, and their functional contributions to both excitatory and inhibitory neurotransmission.

System A transporters as neuronal suppliers of astrocytic glutamine

If astrocytes selectively synthesize glutamine, what transmembrane transporter supplies interneurons with astrocytic glutamine? Because glutamine is an essential amino acid, it is a substrate for multiple amino acid transporters in the solute carrier (SLC) 38 gene family, including Systems A, ASC, L, $b^{0,+}$, $y^{+}L$, and N. Upon functional characterization, Systems A, ASC, L and N were found to mediate the majority of glutamine transport (Broer & Brookes 2001). However, the transporter that supplies neuronal glutamine for GABA synthesis must meet the following requirements: 1) A preference for glutamine transport over other amino acid substrates; 2) Uptake of substrate must define the transport mode; and 3) Neurons should predominantly express the transporter.

Upon cloning and characterizing the properties of individual transporters within the above families, one family meeting the above requirements emerged—the System A

family of SNATs. The neuronal System A members include two subtypes, SNAT1 and SNAT2. Hydropathy plots predict that both SNAT1 and SNAT2 possess 11 transmembrane domains with a cytosolic N-terminus and extracellular C-terminus (Yao *et al.* 2000, Varoqui *et al.* 2000). One distinguishing feature of SNAT1 and SNAT2 is transport of N-methylated substrates which includes the non-metabolizable glutamine analogue, α-(methylamino) isobutyric acid (MeAIB). MeAIB thereby functions as a selective competitive inhibitor for System A transporters. In heterologous cells that express SNAT1 (Varoqui et al. 2000), L-glutamine is the predominant substrate that competes with [^{14}C]MeAIB while alanine is the predominant substrate in cells that express SNAT2 (Yao et al. 2000). This is supported by SNAT1 displaying a higher affinity for L-glutamine (K_M=0.5mM) than SNAT2 (K_M=1.7mM). Furthermore, these K_M's for glutamine transport lie within the concentration range for extracellular glutamine (0.5-1mM) and therefore suggests that these transporters likely operate near saturation.

In addition, both SNAT1 and SNAT2 exhibit electrogenic uptake of glutamine. In oocytes expressing SNAT1 (Mackenzie *et al.* 2003) or SNAT2 (Yao et al. 2000), [^{14}C]MeAIB uptake is sodium-dependent, as replacing NaCl with choline chloride (ChCl) in the extracellular solution abolishes MeAIB uptake. Sodium binding is voltage-dependent and precedes amino acid binding in a 1:1 stoichiometry. The coupling of the electrochemical sodium gradient to amino acid transport enables the generation of steep concentration gradients across the plasma membrane, allowing uptake to serve as the mode of transport for SNAT1 and SNAT2. Lastly, SNAT1 immunoreactivity does not co-localize with GLAST, a marker of astrocytes, in cerebellar granule cell cultures

(Varoqui et al. 2000), in hippocampal cultures (Armano *et al.* 2002), or in hippocampal tissue (Mackenzie et al. 2003). Similar findings are reported for SNAT2 in hippocampal cultures (Armano et al. 2002) and primary cortical cultures or hippocampal tissue (Jenstad *et al.* 2009). Therefore, neurons predominantly express SNAT1 and SNAT2 (Figure 6).

Unlike System A, astrocytes that ensheath both excitatory and inhibitory terminals selectively express the System N family of SNATs, SNAT3 (Boulland *et al.* 2002) and SNAT5 (Cubelos *et al.* 2005). System N co-transports glutamine and 1-Na^+ in exchange for 1-H^+. However, under physiological conditions, SNAT3 transport can reverse, allowing efflux of astrocytic glutamine (Chaudhry *et al.* 1999, Broer *et al.* 2002). This process enables a shuttling of glutamine back to terminals for neuronal uptake (Figure 6).

SNAT1 and SNAT2 neuronal expression and cellular localization

Although neurons appear to selectively express SNAT1 and SNAT2, their cellular localization has called into question their role as suppliers of astrocytic glutamine for neurotransmitter GABA synthesis. Immunohistochemical studies report that both glutamatergic and GABAergic neurons express SNAT1 and SNAT2 protein in adult thalamus, cerebral cortex, brainstem, striatum, olfactory bulb (Conti & Melone 2006, Melone *et al.* 2004, Melone *et al.* 2006), cerebellum (Mackenzie *et al.* 2003, Melone et al. 2004, Gonzalez-Gonzalez *et al.* 2005, Melone et al. 2006), globus pallidus, and regions of substantia nigra (Mackenzie et al. 2003). However, expression could only be detected in neuronal cell somas and dendrites. Likewise, in hippocampus, the majority of

SNAT1 and SNAT2 expression was limited to neuronal cell bodies and dendrites, although synaptic boutons in cultures positively expressed these transporters (Table 1). The absence of strong immunoreactivity of SNAT1 or SNAT2 at synaptic terminals suggests that: 1) SNAT1 and SNAT2 contribute to a metabolic pool of GABA that is not relevant to its neurotransmitter pool or 2) SNAT1 and SNAT2 participate in the glu-gln-GABA pathway at synaptic terminals where their protein expression falls below the current methodological detection limits.

Interestingly, recent studies suggest that in hippocampus, glutamatergic neurons selectively express SNAT2, as weak staining of non-pyramidal neurons was only observed in str. oriens (Table 1). This finding is in direct contrast to the ubiquitous expression on all neurons previously proposed and thus raises an intriguing question: If System A transporters supply astrocytic glutamine for ongoing GABA synthesis, do both transporters serve this role or is this role unique to either SNAT1 or SNAT2?

Table 1. SNAT1 and SNAT2 neuronal expression and cellular localization in hippocampus

Reference	System A subtype	Model system	Cell Type	Neuronal Localization	Co-localization
(Armano et al. 2002)	SNAT1 and SNAT2	Mature cultures	--	Cell soma, dendrites	+MAP2
				Synaptic contacts	+Synaptobrevin
(Mackenzie et al. 2003)	SNAT1	Adult tissue	CA3 pyramidal neurons	Cell soma, dendrites	--
			Dentate granule cells		
			Interneurons of molecular layer	Not in axons or synaptic terminals	
(Gonzalez-Gonzalez et al. 2005)	SNAT2	Tissue	Dentate granule cells	Cell soma, dendrites	--
			Tubular structures in *str. lucidum* of area CA3		
(Jenstad et al. 2009)	SNAT2	Adult tissue	CA1 and CA3 pyr. neurons	Cell soma, dendrites	+MAP2 (granule cells)
			Dentate granule cells		
			Afferents in *str. luciduum*	Not in axons or synaptic terminals	-Neurofilaments (granule cell layer)
			Weak staining of non-pyramidal neurons in *st. oriens* only		

SNAT1 and SNAT2 regulation

Most of our knowledge regarding the regulation of System A transporters stems from studies in non-neuronal systems, leaving a significant deficit in our understanding of their unique regulation in neurons. Early studies in non-neuronal cells and tissues demonstrated that growth factors (Ensenat *et al.* 2001), hypertonicity (Alfieri *et al.* 2001), and insulin (Hyde *et al.* 2002) stimulate SNAT2-mediated uptake of various amino acids. The mechanism required either *de novo* synthesis of SNAT2 protein (Ensenat et al. 2001, Alfieri et al. 2001) or insertion of existing transporters at the cell surface (Hyde et al. 2002). In neocortical neuronal cultures, chronic deprivation (8h) of amino acids increases SNAT2, but not SNAT1, uptake activity due to increased *de novo* synthesis of SNAT2 protein (Grewal *et al.* 2009). In the same system, a one-hour treatment with brain-derived neurotrophic factor (BDNF) stimulates SNAT1 activity by increasing mRNA and protein expression (Burkhalter *et al.* 2007). Interestingly, shortening the BDNF incubation to twenty minutes stimulates SNAT1 uptake activity without enhancing protein expression, suggesting a possible translocation of SNAT1 from an intracellular compartment to the cell surface. The previous two studies demonstrate that neurons can uniquely regulate SNAT1 and SNAT2 uptake activity in order to meet specific metabolic demands. In addition, both SNAT1 and SNAT2 are negatively regulated by protons (Chaudhry et al. 2002) and possess sites for posttranslational modifications, including three PKC consensus sequences (N-terminus and an intracellular loop) and two extracellular glycosylation sites (Mackenzie & Erickson 2004). However, whether these posttranslational modifications regulate System A transport activity remains unknown.

SNAT1 and SNAT2 function in excitatory synaptic transmission

Given that both glutamatergic and GABAergic neurons require a supply of glutamate to maintain their neurotransmitter pools, one might expect that SNAT1 and SNAT2 also supply astrocytic glutamine for neurotransmitter glutamate synthesis. Alternatively, given that glutamatergic neurons appear to selectively express SNAT2 in hippocampus (Jenstad et al. 2009, Gonzalez-Gonzalez et al. 2005), SNAT1 and SNAT2 may play unique roles in the synthesis of neurotransmitters GABA and glutamate. Although future chapters will focus on the role of System A transporters in modulating inhibitory neurotransmission, I will provide a brief background of their controversial role in excitatory neurotransmission in order to provide context for future points of discussion.

Several studies have examined if glutamine supplied by System A transporters contributes to neurotransmitter glutamate synthesis and synaptic release. In acute rat hippocampal slices (P12-24), L-glutamine (4mM) increased the concentration of glutamate in the synaptic cleft and thus mEPSC amplitudes recorded from CA1 pyramidal neurons. However, this increase only occurred at glutamine incubation times greater than 4 hours (Kam & Nicoll 2007). Glutamine supplied by System A transporters mediated the delayed effect, as MeAIB (25mM) in the presence of L-glutamine (4mM) prevented the increase in mEPSC amplitude. This timecourse suggests that glutamine does not contribute to ongoing glutamate synthesis and maintenance of acute excitatory neurotransmission.

However, recent studies demonstrate that glutamatergic neurons require glutamine to sustain the excessive excitability in various *in vitro* models of seizure-like

activity. In horizontal thalamic slices from juvenile rats (Bryant *et al.* 2009) or in hippocampal slices from adult rats (Bacci *et al.* 2002), blockade of postsynaptic $GABA_A$ receptors induces spontaneous epileptiform oscillations (bursts of action potentials at defined frequencies) mediated by initial activation of ionotropic glutamate receptors. Sustaining this hyperexcitability required glutamine, as MeAIB (5-12.5mM) reduced either the duration (Bryant et al. 2009) or frequency (Bacci et al. 2002) of these epileptiform oscillations. Similarly, in neocortical slices, 10mM MeAIB blocked the polyphasic activity of evoked field potentials that characterizes the hyperexcitability observed in the neocortical undercut model of epilepsy (Tani *et al.* 2007). Tani and colleagues concluded that cortical excitatory neurons increase protein expression of both SNAT1 and SNAT2 to mediate the increased glutamine requirement, although only the amount of SNAT2 protein appears increased in immunoblots.

Taken together, it appears that glutamatergic neurons may only require glutamine to synthesize glutamate under pathological conditions of increased metabolic demand. In addition, SNAT2 may play a unique role as the main System A transporter on glutamatergic neurons mediating this requirement.

SNAT1 and SNAT2 function in inhibitory synaptic transmission

Similar to the immunohistochemical data, results from several studies indicate that the functional role of System A transporters in the glu-gln-GABA pathway remains controversial. First, in adult guinea pig cortical slices under depolarizing conditions (40mM KCl), 10mM Histidine, but not 10mM MeAIB, inhibits the labeling of glutamate, glutamine, and GABA from [^{13}C]-glucose measured using NMR spectroscopy (Rae *et al.*

2003). The authors conclude that during prolonged periods of synaptic activity, astrocytic glutamine contributes to synaptic GABA through uptake by a non-System A glutamine transporter that remains sensitive to histidine. However, this study does not eliminate the possibility that glutamine synthesized from uptake of synaptically released glutamate by EAAT2 on astrocytes (v. *de novo* synthesis of glutamate from glucose) is shuttled to inhibitory synapses for uptake by System A transporters.

Second, in adult rat hippocampal slices, 5mM MeAIB did not reduce mIPSC amplitudes in the absence of synaptic activity, suggesting that glutamine does not maintain basal GABA synthesis. However, applying a moderate burst stimulation protocol (4 pulses of 50Hz, repeated every 20s for 15m) beneath (20-50μm) the CA1 pyramidal layer in stratum rad. acutely increased evoked IPSC (eIPSC) amplitudes recorded from CA1 pyramidal neurons (Liang *et al.* 2006). This increase required astrocytic glutamine, as both 5mM MeAIB and 1.5mM MSO independently reduced post-stimulation eIPSC amplitudes below pre-stimulation amplitudes. The authors conclude that this stimulation increased flux of glutamine through the glu-gln-GABA pathway, but they did not explore whether the entire cycle or individual processes within the pathway were upregulated to mediate the mechanism. They do demonstrate that the increase in control eIPSC amplitudes was partly mediated by increasing the release probability of GABAergic vesicles, but this increase was sustained in the presence of both MeAIB and MSO.

Lastly, applying a high frequency stimulation (HFS) protocol (100Hz for 1s) again beneath the CA1 pyramidal layer in str. rad. of adult mouse hippocampal slices acutely increased mIPSC amplitudes recorded from CA1 pyramidal neurons (Hartmann

et al. 2008). In contrast to the previous study, this increase did not require glutamine, as 5mM MeAIB did not reduce the stimulated increase in mIPSC amplitudes. Instead, pharmacological blockade of glutamate uptake combined with GABA reuptake by GAT-1 eliminated the stimulated increase. The authors concluded that HFS enhanced the release of neurotransmitter glutamate and GABA available for uptake at inhibitory terminals (and presumably vesicular GABA content) although the authors did not provide any data to support this mechanism. In addition, electrical stimulation in str. rad. probably stimulated only a subpopulation of interneuron terminals, while multiple populations of interneurons likely contributed to the population of mIPSCs recorded. Therefore, the hypothesized mechanism above may not accurately reflect the cellular processes that mediate the increase in synaptic GABA at the subpopulation of stimulated interneuron terminals. Taken together, glutamine's contribution to activity-dependent GABA synthesis remains controversial and possibly reflects the different approaches applied to increase neuronal activity.

In summary, whether System A transporters supply glutamine to maintain ongoing GABA synthesis constitutively or in an activity-dependent manner remains unresolved. However, because glutamine exists at high extracellular concentrations that are ubiquitously available to interneuron terminals, constitutive glutamine uptake is a likely mechanism by which the majority of interneurons can maintain ongoing GABA synthesis. Furthermore, if System A transporters operate at saturation as biochemical studies suggest, up-regulating the surface activity of System A transporters may be an effective mechanism to increase neuronal glutamine supply and thus enhance GABA synthesis during periods of increased neuronal demand.

Plasticity at Hippocampal Inhibitory Synapses

The previous section outlined the controversial role for System A transporters as neuronal suppliers of astrocytic glutamine for GABA synthesis. It also demonstrated our lack of understanding of System A transport function and regulation on interneurons. Regardless, in order for modulation of System A transport activity to dynamically regulate GABA synthesis and vesicular GABA content, inhibitory synapses must exhibit plastic properties. Synaptic plasticity represents the process by which different stimuli or cellular signals modify the structure or function of synapses. Long-Term Potentiation (LTP) and Long-Term Depression (LTD) increase and decrease synaptic strength, respectively, and thus represent one model of synaptic plasticity. In addition, synapses possess short-term mechanisms of synaptic plasticity that allow neurons to acutely adapt to a range of activity states. Like excitatory synapses, inhibitory synapses appear to be remarkably plastic. In this brief section, I will provide examples of LTP_{GABA} and LTD_{GABA} at both developing and mature inhibitory synapses to illustrate this point.

Plasticity at interneuron synapses on CA1 pyramidal neurons

Within a restricted time window during hippocampal maturation (P0-P8), delivering "depolarizing pulses" [DPs; 20 pulses (500ms each, -80/-90 to 0mV step) at 0.1Hz] to CA3 pyramidal neurons induced a long-lasting (>30m) increase in spontaneous $GABA_A$ receptor-mediated post-synaptic current ($sGABA_A$-PSC) amplitudes and frequency (Gubellini *et al.* 2001). In addition, DPs enhanced miniature $GABA_A$-PSC frequency but not amplitude, suggesting that DPs increase the probability of GABAergic

vesicle release at individual inhibitory terminals. This enhanced release of synaptic GABA activates $GABA_A$ receptors to efflux chloride, depolarize the postsynaptic membrane and activate voltage-gated calcium channels. This process triggers calcium influx, a requirement for this form of LTP_{GABA} (Caillard *et al.* 1999). LTP_{GABA} induction also requires activation of presynaptic Tropomyosin-related kinase receptor B (TrKB) through its endogenous ligands, BDNF or neurotrophin-4 (Gubellini *et al.* 2005). The authors propose that the triggered calcium influx from $GABA_A$ receptor activation likely stimulates endogenous BDNF release, which in turn activates presynaptic TrkB receptors at interneuron terminals to increase synaptic release of GABA. This example of LTP_{GABA} expressed at immature inhibitory synapses presents one possible activity-dependent mechanism by which GABA facilitates synaptogenesis and hippocampal maturation.

In adult hippocampal slices, afferent fibres within str. rad. stimulated at theta-burst (5 bursts of 4 stimuli at 100Hz, 200ms apart) frequencies induced a long-lasting increase in evoked IPSC (eIPSC) amplitudes recorded from CA1 pyramidal neurons (Patenaude *et al.* 2003). LTP_{GABA} was not associated with a change in the paired-pulse ratio, consistent with a postsynaptic locus of expression that does not involve changes in GABA release. Blockade of $GABA_B$ or group I/II mGluRs each partially reduced LTP_{GABA}, while introducing the calcium chelator BAPTA into the recording pipette completely blocked LTP_{GABA}. These findings suggested that induction of LTP_{GABA} involved calcium influx postsynaptically, although the precise molecular signals were not explored.

Adult interneuron-CA1 pyramidal neurons synapses are also capable of eliciting long-term depression (LTD_{GABA}) through a heterosynaptic mechanism involving neighboring glutamatergic terminals. High frequency (2 trains of 100 stimuli at 100Hz, 20s apart) or theta-burst (10 bursts of 5 stimuli at 100Hz, 200ms apart) stimulation of afferent fibres within str. rad. induced a long-lasting reduction in eIPSC amplitudes recorded from CA1 pyramidal neurons (Chevaleyre & Castillo 2003). LTD_{GABA} was associated with an increase in the paired-pulse ratio after HFS and thus consistent with a presynaptic reduction in evoked GABA release. Interestingly, stimulating afferent fibres within the str. pyramidale layer failed to induce LTD_{GABA}, consistent with a heterosynaptic mechanism between glutamatergic and GABAergic neurons that form synapses on distal CA1 apical dendrites. Subsequent pharmacological experiments revealed that synaptically released glutamate activates postsynaptic group I mGluRs to increase downstream diacylglycerol and consequently 2-AG production. 2-AG functions as a retrograde signal, where it transverses the plasma membrane and synaptic cleft to activate presynaptic endocannabinoid receptors (CB1) expressed at interneuron terminals. Activation of CB1 receptors can then decrease GABA release short-term through inhibition of voltage-gated calcium channels or long-term through reduced PKA-mediated signaling involving the active zone protein RIM1α (Chevaleyre *et al.* 2007).

Plasticity at glutamatergic synapses on interneurons

Multiple studies have identified mechanisms of long-term plasticity at glutamatergic synapses on area CA1 interneurons that involve both NMDA receptor-dependent and -independent mechanisms (Kullmann & Lamsa 2007). In P21-P28

hippocampal slices, paired stimulation of schaffer-collaterals (2Hz for 1m) with post-synaptic depolarization of str. rad. interneurons resulted in a NMDA receptor-dependent potentiation of the EPSP initial slope (a hallmark of LTP) (Lamsa *et al.* 2005). LTP_{GABA} occurred in a small proportion interneurons that participate in feedforward inhibition and thus may serve as a mechanism to counterbalance excessive excitability at schaffer collateral-CA1 pyramidal neuron synapses. Conversely, HFS (5 pulses at 100Hz, repeated 20 times) of local CA1 axon collaterals paired with post-synaptic hyperpolarization of str. oriens interneurons resulted in LTP_{GABA} that required calcium-permeable AMPA receptor activation (Lamsa *et al.* 2007). This form of LTP_{GABA} occurred robustly in interneurons that participate in feedback inhibition and thus may limit CA1 pyramidal firing.

LTP and LTD define traditional mechanisms of synaptic plasticity that alter presynaptic vesicle release probability or postsynaptic receptor responsiveness. Alternatively, presynaptic control of quantal size has gained recent attention as an additional mechanism of synaptic plasticity (Edwards 2007), yet mechanisms that underlie this form of synaptic plasticity at hippocampal interneuron terminals remain unresolved.

Section Summary, Hypotheses and Central Questions

In hippocampus, GABA serves multiple roles during development and throughout adulthood, which include: 1) orchestrate synapse maturation and synaptogenesis, 2) maintain inhibitory synaptic transmission, and 3) regulate the excitability and synchronize the neuronal output of principal neurons. The previous section demonstrated that inhibitory synapses are indeed plastic, yet we know very little about presynaptic mechanisms that adapt GABA levels during the wide range of developmental and activity states an interneuron experiences. In addition, it is now apparent that a functional coupling exists between GABA synthesis and vesicle filling and that neurons require a supply of precursor glutamate to maintain GABA synthesis and vesicle content. Therefore, transmembrane transporters that supply glutamate, or glutamine, provide an effective presynaptic mechanism to maintain or adapt GABA levels in an activity-dependent manner. However, the hippocampus' unique anatomical features likely prevent direct glutamate uptake by EAAT3 to maintain constitutive GABA synthesis, and moreover, may only provide an adaptive mechanism to augment GABA synthesis at a sub-population of inhibitory terminals.

Because glutamine is ubiquitously available to inhibitory terminals, I hypothesize that glutamine uptake by SNAT1 and/or SNAT2, with its subsequent conversion to glutamate and GABA, defines an additional, independent pathway to maintain constitutive GABA synthesis. Since System A transporters likely operate near saturation, I also hypothesize that up-regulation of System A surface activity provides interneurons with an effect presynaptic mechanism to augment GABA synthesis, vesicular GABA

content, and inhibitory synaptic strength in response to increased neuronal demand. Lastly, because GABA's role changes throughout postnatal development, I hypothesize that the potential constitutive or activity-dependent roles of System A transporters may be developmentally regulated to link the activity-related demands of GABA to its rate of synthesis. The questions asked in Chapters II and III that drove the above hypotheses are as follows:

In Chapter II, I asked the following questions: Do System A transporters constitutively supply inhibitory terminals with glutamine to maintain ongoing GABA synthesis and inhibitory synaptic transmission? If so, does this supply of glutamine exist in a dynamic equilibrium with GABA synthesis and vesicle filling, similar to glutamate supplied by EAAT3? Moreover, what are the cellular processes upstream of System A transporters that supply glutamine to inhibitory terminals?

In Chapter III, I asked two central questions: 1) Does neuronal activity upregulate the surface activity of System A transporters in order to increase glutamine supply, vesicular GABA synthesis and inhibitory synaptic transmission during periods of increased metabolic demand? and 2) Are either the potential constitutive or activity-dependent contributions of System A-mediated glutamine uptake to synaptic GABA developmentally regulated? If so, what is the mechanism and does it uniquely involve one System A subtype (SNAT1 or SNAT2)?

CHAPTER II

GLUTAMINE UPTAKE BY SYSTEM A TRANSPORTERS MAINTAINS NEUROTRANSMITTER GABA SYNTHESIS AND INHIBITORY SYNAPTIC TRANSMISSION

Introduction

To maintain inhibitory neurotransmission in the brain, GABA must be supplied to recycling synaptic vesicles. Inhibitory neurons express GABA transporters, but there is no evidence that reuptake of transmitter is required to maintain the synaptic pool of GABA. Alternatively, inhibitory neurons are capable of GABA synthesis from glutamate using glutamic acid decarboxylase (GAD). They must, however, have a supply of glutamate because neurons are not capable of ongoing glutamate production (Bak *et al.* 2006). The high affinity sodium-dependent glutamate transporter EAAT3 is expressed by interneurons of the hippocampus (Berger & Hediger 1998, Kugler & Schmitt 1999) and is observed on inhibitory synaptic terminals (He et al. 2000, Rothstein *et al.* 1994). Knockdown of EAAT3 *in vivo* reduces GABA synthesis and results in spontaneous epileptic seizures (Sepkuty et al. 2002). Electrophysiological studies show that the vesicular content of GABA is rapidly altered in response to changes in glutamate uptake (Mathews & Diamond 2003), demonstrating that glutamate supply to neurons regulates neurotransmitter GABA synthesis, which in turn regulates vesicular filling and synaptic strength.

Biochemical studies (Paulsen et al. 1988, Battaglioli & Martin 1991, Sonnewald et al. 1993, Battaglioli & Martin 1996, Waagepetersen *et al.* 1999, Rae et al. 2003) indicate that glutamine also may contribute to neurotransmitter GABA synthesis. When examined in hippocampal slices, exogenously applied glutamine does not enhance GABA release (Szerb & O'Regan 1984), but endogenous glutamine content of slices decreases over time and this decline is followed by a slow (over 3 to 6 hours) reduction in GABA levels (Kapetanovic et al. 1993). Although there are several transporters that are capable of mediating uptake of glutamine by neurons, SNAT1 and SNAT2, comprising the System A subfamily of sodium-coupled neutral amino acid transporters, appear to be the most important. They are expressed in excitatory and inhibitory neurons (Reimer *et al.* 2000, Mackenzie et al. 2003, Melone *et al.* 2004, Melone *et al.* 2006), are observed at synaptic contacts in hippocampal neurons in culture (Armano et al. 2002) and therefore have been suggested to supply substrate for neurotransmitter synthesis. Once taken up, glutamine conversion to glutamate is thought to be catalyzed by phosphate-activated glutaminase type 1 (GLS1) (Kvamme & Olsen 1980). However, GLS1 expression in GABAergic neurons has not been shown, and knockout of this gene in mice impaired glutamatergic but not GABAergic synaptic transmission (Masson *et al.* 2006). Moreover, recent biochemical and immunohistochemical studies have argued against a role for System A transporters in neurotransmitter metabolism (Rae et al. 2003, Melone et al. 2004, Conti & Melone 2006). In one study, glutamine uptake was shown to augment inhibitory synaptic transmission under certain stimulation protocols (Liang et al. 2006), suggesting that the glutamine supply may be required for GABA synthesis only when demand reaches a critical level.

Glutamate released from excitatory synapses is normally taken up by astrocytes that express high levels of glutamate transporters, primarily EAAT2, and is subsequently converted to glutamine by glutamine synthetase (GS) (Martinez-Hernandez *et al.* 1977). Astrocytes express the System N glutamine transporters, SNAT3 and SNAT5, which have been proposed to transfer glutamine back to neurons by reverse transport (Chaudhry et al. 1999, Broer & Brookes 2001, Cubelos et al. 2005). Since neuronal glutamate transporters regulate the synaptic pool of GABA in hippocampal interneurons (Mathews & Diamond 2003), we sought to determine the role of System A glutamine transporters in the synthesis of GABA at inhibitory synapses. Our results demonstrate that glutamine transporters on inhibitory neurons of the hippocampus contribute to the supply of glutamate required for GABA synthesis. IPSC and mIPSC amplitudes were rapidly reduced in response to interrupting the glutamine supply from astrocytes, consistent with a reduction in the GABA content of synaptic vesicles. Our data demonstrate that a constitutive supply of glutamine from astrocytes is necessary to maintain inhibitory synaptic transmission even under resting conditions.

Experimental Procedures

Materials

MeAIB, DL-threo-b-hydroxyaspartic acid (THA), L-histidine, L-methionine sulfoximine (MSO), 6,7-dinitroquinoxaline-2,3-dione (DNQX) and dizocilpine maleate (MK-801) were obtained from Sigma (St. Louis, Missouri, USA). Dihydrokainate (DHK) and TTX were obtained from Tocris (Ellisville, Missouri, USA).

Preparation of hippocampal slices and electrophysiological recording

Seven to fourteen day old Sprague Dawley rats were deeply anesthetized with isoflurane and decapitated under a protocol approved by Vanderbilt University's Institutional Animal Care and Use Committee. Hippocampi were removed and cut into 400 μm sections (Vibratome Company, St. Louis, MO) in an ice-cold oxygenated solution containing (in mM): sucrose 75, NaCl 87, KCl 2.5, $MgCl_2$ 7, $CaCl_2$ 0.5, NaH_2PO_4 1.25, glucose 25, and $NaHCO_3$ 25. Slices were transferred to a chamber containing artificial cerebrospinal fluid (ACSF), which contained (in mM): NaCl 119, $CaCl_2$ 2.5, KCl 2.5 $MgCl_2$ 1.3, Na_2PO_4 1, $NaHCO_3$ 26.2 and glucose 11, (pH 7.4, 290 mOsm) and bubbled with 95% O_2/5% CO_2. Slices were incubated at 35^{o}C for 30 minutes, then at room temperature for 30 minutes before recording.

Patch pipettes with resistances of 3-5 MΩ were filled with a solution containing (in mM): cesium chloride 130, HEPES 10, EGTA 10, lidocaine N-ethyl bromide (QX-314) 1, Na^{2+}-ATP 0.2, and Mg^{2+}-ATP 2 (pH 7.4, 290 mOsm). Whole cell recordings were obtained at a holding potential of –60 mV with a MultiClamp 700A amplifier

(Molecular Devices Corporation, Sunnyvale, CA). Access resistance was monitored throughout the experiment and data were discarded if increase was more than 20%. Slices were constantly perfused at approximately 2 mL/min with ACSF (bubbled with 95% O_2/5% CO_2) containing DNQX (10 μM) and MK-801 (5 μM) to block postsynaptic glutamate receptors. After control currents were obtained, a five-minute period of perfusion with drug occurred before the drug effect was evaluated. Previous studies have shown that the effects of transporter (Mathews & Diamond 2003) and enzyme (Cavelier & Attwell 2005) inhibitors on neurotransmitter metabolism occur within five minutes. Recovery of responses similarly was assessed after a five-minute washout period. IPSCs were evoked with a bipolar stainless steel electrode placed in stratum oriens (constant current stimulation, 25-100 mA, 100 ms duration). For experiments evaluating the effect of MeAIB on post-synaptic GABA receptors, GABA (10 mM) was pressure-applied from a glass pipette in ACSF alone and during perfusion of the slice with 10 mM MeAIB. Spontaneous miniature IPSCs (mIPSCs) were obtained in the presence of 0.5μM tetrodotoxin (TTX). For "evoked" mIPSC experiments, hyperosmolar sucrose (500 mM in ACSF containing TTX, DNQX and MK-801) was pressure applied for 3 seconds at two-minute intervals with a glass pipette near the neuron soma. All experiments were performed at room temperature unless otherwise indicated.

Data analysis

For IPSC analysis, 15 responses were averaged in each condition to determine the peak current, and the drug effect in multiple cells was assessed using the paired t-test. For mIPSC analysis, individual events meeting threshold criteria were detected using

MiniAnalysis software (Synaptosoft, Fort Lee, NJ, USA) and visually inspected to exclude artifacts. Those single events arising from a stable baseline were used for analysis. At least 100 events were recorded from each cell in each condition, and the median was calculated. Each experiment was performed in multiple cells ('n'; range 5-9) prepared from at least 3 animals and the mean of the medians ± standard deviation was reported for each condition. To assess the drug effects, changes in median amplitudes during drug application were reported as a percent change from baseline ± SEM. Data were tested for normality (Kolmogorov-Smirnov test) and, unless otherwise noted, passed the normality test and were compared using a paired t-test. Cumulative probability histograms were generated by averaging (from multiple cells) the distribution of mIPSC amplitudes, normalized to the mean control mIPSC amplitude for each cell. For repeated measures of mIPSC amplitudes in different conditions (i.e. experiments depicted in figure 6), one-way repeated measures ANOVA was used.

Results

To determine the role of glutamine uptake by neurons in the synthesis of GABA destined for synaptic release, we examined the effect of α-(methyl-amino) isobutyric acid (MeAIB), a selective system A transport inhibitor (Broer & Brookes 2001), on IPSCs recorded from CA1 pyramidal neurons in hippocampal slices. With an apparent affinity of 1.1 mM (Mackenzie et al. 2003), MeAIB has been used at 5-50 mM to inhibit System A transporters in a variety of *in vitro* studies (Bacci et al. 2002, Rae et al. 2003, Liang et al. 2006). MeAIB (10 mM) reduced electrically evoked IPSCs by 37 ± 9 % of control ($p<0.001$, n=13 cells; Figure 7A) without significantly changing the paired-pulse ratio (PPR was 110 ± 5 % of control; $p=0.09$; Figure 7B,C), consistent either with a reduction in [GABA] in the synaptic cleft without a change in release probability, or with a post-synaptic effect on $GABA_A$ receptors. To address the latter possibility, we tested the effect of MeAIB (10 mM) on the response to pressure-applied GABA. MeAIB did not alter the peak current elicited by GABA (10 mM) on CA1 pyramidal neurons (current in MeAIB was 97 ± 2 % of control; n=3; Figure 7D), demonstrating that MeAIB did not affect $GABA_A$ receptors at this concentration.

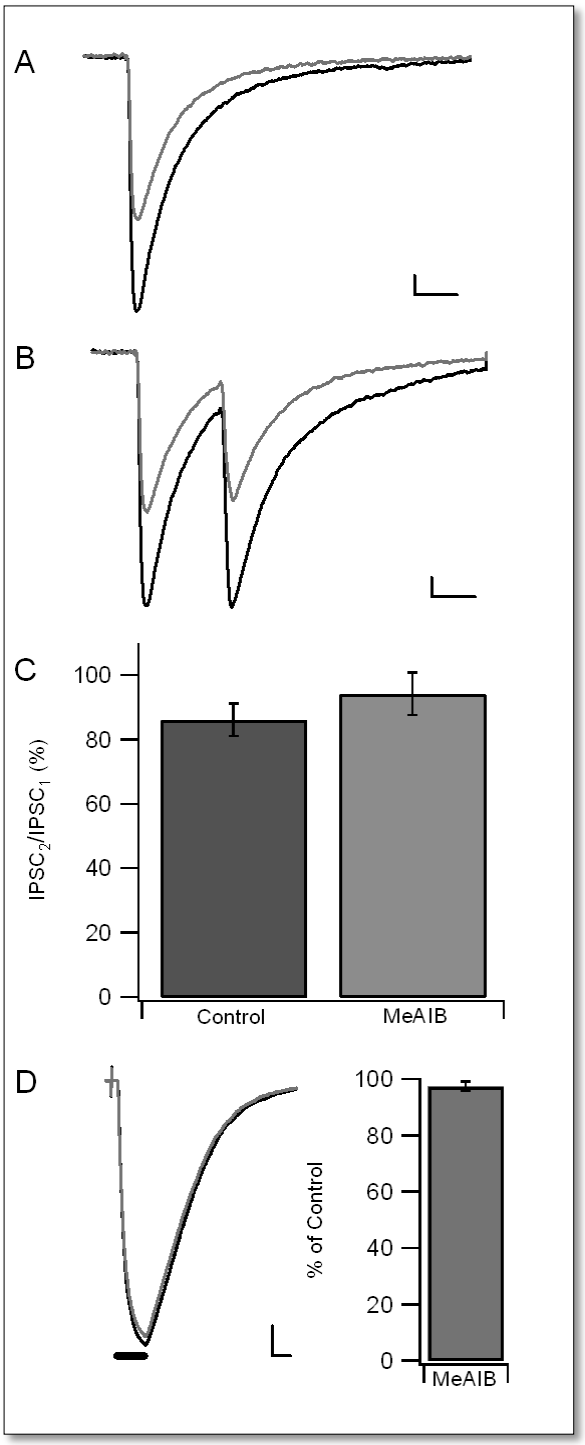

Figure 7. MeAIB reduces electrically-evoked IPSCs without altering paired-pulse ratio or blocking postsynaptic $GABA_A$ receptors

Figure 7. MeAIB reduces electrically-evoked IPSCs without altering paired pulse ratio or blocking post-synaptic $GABA_A$ receptors

A, Electrically evoked IPSC in control solution (black) and in the presence of the SNAT1 inhibitor, MeAIB (10 mM, gray). **B,** IPSCs evoked by a pair of stimuli 50 ms apart in control solution (black) and in 10 mM MeAIB (gray). Scale bars in **A** and **B** represent 100 pA and 25 ms. **C,** Comparison of the paired pulse ratio ($IPSC_2/IPSC_1$) in control (black) and MeAIB (gray). Error bars indicate SEM. **D**, Representative currents elicited by application of GABA (10 mM) in control (black) and in the presence of MeAIB (10 mM, gray). Bar beneath traces indicates duration of GABA application. Scale bars represent 500 pA and 200 ms. Graph represents mean response ± SEM in the presence of MeAIB as a percent of control response.

To investigate the alternative that MeAIB reduced GABA release, we examined its effect on responses to the release of single vesicles. Median miniature IPSCs (mIPSCs) amplitudes were reduced by 16 ± 5% (from 32.7 ± 8.2 pA to 27.3 ± 8.0 pA: p = 0.01, n = 8 cells; Figure 8A-C) after 5 minutes of perfusion with MeAIB. When examined, the change was reversible after drug washout (median amplitude in recovery compared with MeAIB; p=0.005; n=4 cells; see Figure 8B). The frequency of mIPSCs was unchanged; the median inter-event interval was 1.2 ± 0.9 s in control and 1.5 ± 1.0 s in MeAIB (p=0.15, Wilcoxon Signed Rank test, n = 9 cells; Figure 8D). Together with its lack of effect on paired pulse ratio, these data showing that MeAIB alters both IPSC and mIPSC amplitudes without altering the probability of vesicle release are consistent with a reduction in vesicular content of GABA.

Because the activity of transporters and enzymes are highly sensitive to temperature, we examined the effect of MeAIB at 34°C and found no difference in the effect of MeAIB on mIPSC amplitudes compared with room temperature. Median mIPSC amplitudes were reversibly reduced 15 ± 4 % (from 36.1 ± 7.6 pA to 30.4 ± 5.8 pA: p = 0.005, n = 9 cells; recovery compared with MeAIB: p=0.03, n = 8; Figure 9A,C). Additionally, because non-specific effects of drugs may occur at high concentrations, we evaluated the effect of histidine, a non-selective inhibitor of both System A and System N transporters (Broer & Brookes 2001). Similar to MeAIB, histidine (10 mM) reduced median mIPSC amplitudes by 21 ± 5 % (from 34.5 ± 6.8 pA to 27.1 ± 6.0 pA: p = 0.02, n = 6 cells; Figure 9B,C).

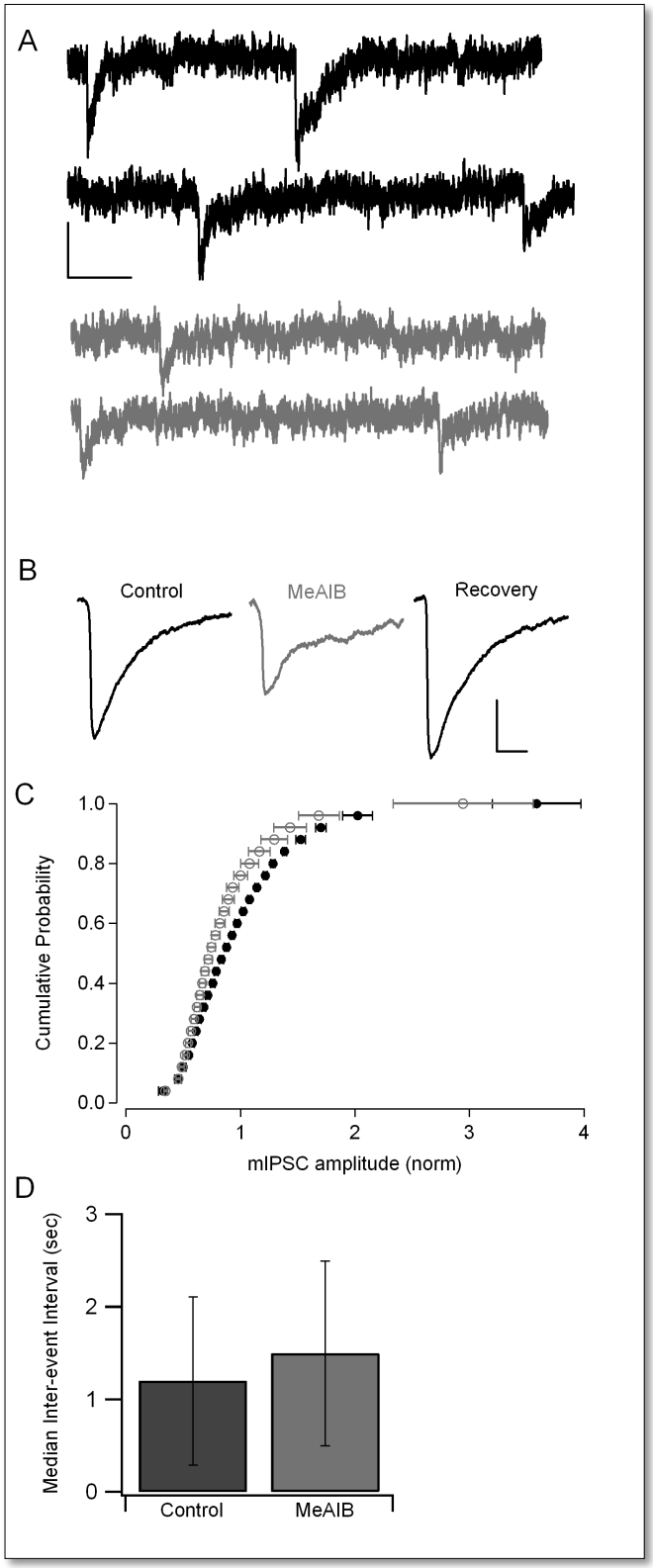

Figure 8. **MeAIB reduces mIPSC amplitudes but does not alter frequency**

Figure 8. **MeAIB reduces mIPSC amplitudes but does not alter frequency**
A, Sample current traces showing continuous recordings in control solution (black) and after 5 minutes in 10 mM MeAIB (gray). Scale bars represent 25 pA and 25 ms. **B,** Average mIPSC waveforms from a representative cell in control, MeAIB and after drug washout. Scale bars represent 10 pA and 20 ms. **C,** Cumulative probability histograms of normalized mIPSC amplitudes in control solution (black symbols) and in the presence of MeAIB (gray symbols). Data were averaged from 8 cells and error bars indicate SEM. **D,** Comparison of median inter-event interval in control (black symbols) and MeAIB (gray symbols). Error bars indicate SD.

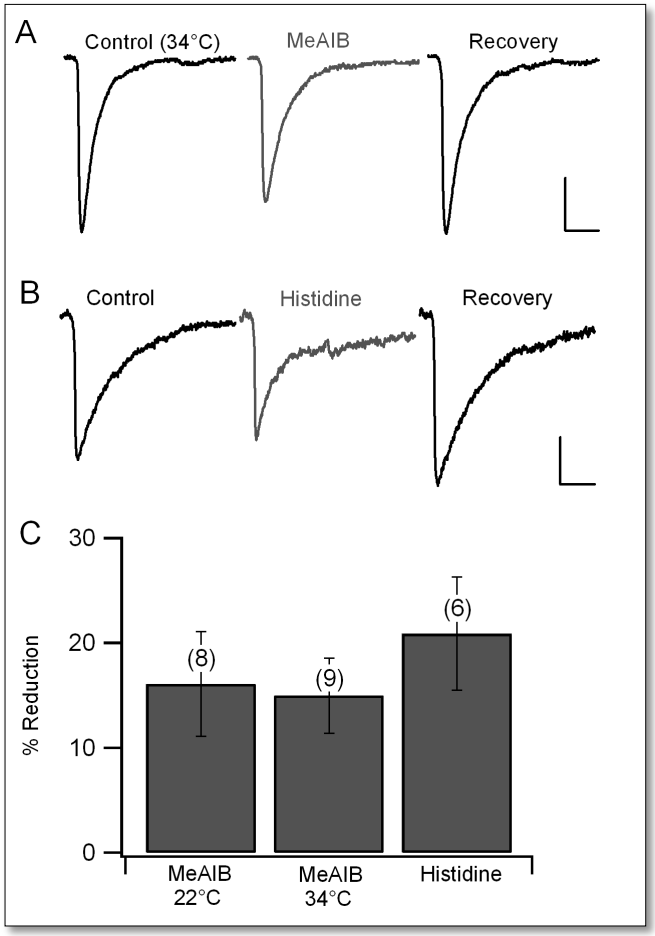

Figure 9. Effect of MeAIB on mIPSC amplitudes is reproduced at physiological temperature and with histidine

A, Average mIPSC waveforms from a representative cell recorded at 34°C in control (black), in 10 mM MeAIB (gray) and after drug washout. **B,** Average mIPSC waveforms from a representative cell in control (black), in 10 mM histidine (gray) and after drug washout. Histidine is a non-selective inhibitor of SNATs. Scale bars represent 10 pA and 20 ms in both panels. **C,** Comparison of the effect of MeAIB at room and physiological temperatures with the effect of histidine on median mIPSC amplitudes. Number of cells is shown in parentheses. Error bars indicate SEM.

Since mIPSCs occur spontaneously in the absence of neuronal activity, we next asked whether these recorded events represent a pool of vesicles that is metabolically distinct from those released following an action potential. To address this question, we used hypertonic solution to evoke release of vesicles comprising the readily releasable pool (Rosenmund & Stevens 1996). The effect of MeAIB on hyperosmotically-evoked mIPSC amplitudes was identical to its effect on spontaneous mIPSCs recorded in the same cells ($p=0.5$; $n = 3$ cells; Figure 10).

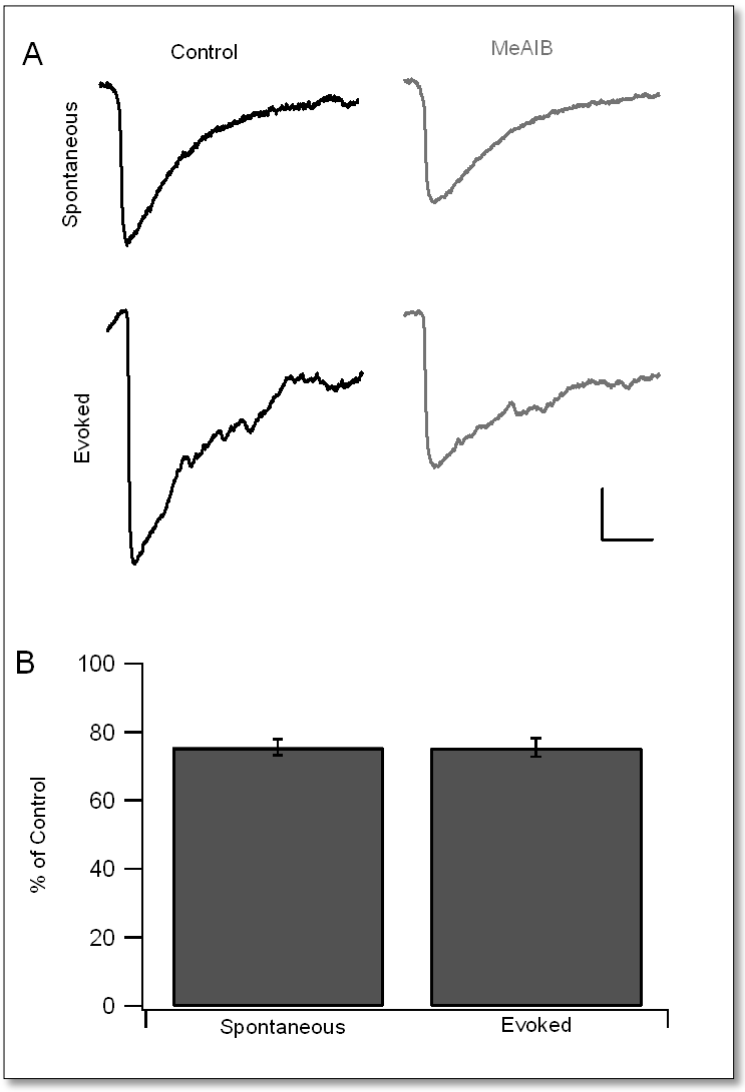

Figure 10. MeAIB reduces both spontaneous and hyperosmotically-evoked mIPSCs
A, *Upper panel,* Average spontaneously occurring mIPSC waveform in control solution (black) and in 10 mM MeAIB (gray) from a representative cell. *Lower panel,* Average evoked mIPSC waveform during pressure application of 500 mM sucrose in control solution (black) and in MeAIB (gray) recorded in the same cell as the panel above. Scale bars represent 10 pA and 20 ms. **B,** Comparison of the effect of MeAIB on spontaneous and evoked mIPSC amplitudes. Error bars indicate SEM.

Previous studies demonstrated that exogenous glutamate increases mIPSC amplitudes via an effect of GABA synthesis (Mathews & Diamond 2003). Therefore, we asked whether addition of exogenous glutamine would increase mIPSC amplitudes. Addition of glutamine (500 mM or 2 mM) to the perfusate had no effect on mIPSC amplitudes (39.8 ± 8.8 pA vs. 39.9 ± 11.4 pA: $p = 1.0$, $n = 5$ cells; Figure 11), suggesting that the extracellular concentration of glutamine within the slice is sufficient to saturate neuronal glutamine uptake. Since glutamine normally is absent from our perfusion solutions, we hypothesized that saturating levels of glutamine are maintained by astrocytes that take up extracellular glutamate to produce glutamine. If this glutamate-glutamine cycle mediated by astrocytes were the source of glutamine (Figure 12A), then we hypothesized that MeAIB would not reduce mIPSC amplitudes after the supply of glutamine from astrocytes was interrupted. In the presence of L-methionine sulfoximine (MSO; 5 mM), an inhibitor of astrocytic glutamine synthetase, or of dihydrokainate (DHK; 300 μM), an inhibitor of the predominant astrocytic glutamate transporter EAAT2, MeAIB had no effect on mIPSC amplitudes ($p=0.11$, $n = 8$ for MSO; $p=0.58$, $n=5$ for DHK; Figure 12A, B). Interestingly, DHK and MSO alone did not produce a reduction as would be expected if they only reduced glutamine supply from astrocytes to neurons. However, unlike MeAIB, by impairing glutamate clearance by astrocytes, both MSO (Cavelier & Attwell 2005) and DHK (Munoz *et al.* 1987) raise extracellular glutamate levels. We hypothesized that increased neuronal glutamate uptake, presumably via EAAT3 (see Figure 12A), counteracted the effect on GABA synthesis of DHK and MSO. To test this hypothesis, we co-applied the non-selective EAAT inhibitor THA (300 mM) with DHK to eliminate EAAT3-mediated uptake, and observed a 17 ± 2 %

reduction in mIPSC amplitudes ($p<0.001$; $n = 5$ cells, Figure 12C). Therefore, in support of the model whereby both glutamine and glutamate uptake each contribute to neurotransmitter GABA synthesis, the inhibition of EAAT3 in addition to EAAT2 resulted in a reduction in mIPSC amplitudes comparable to MeAIB.

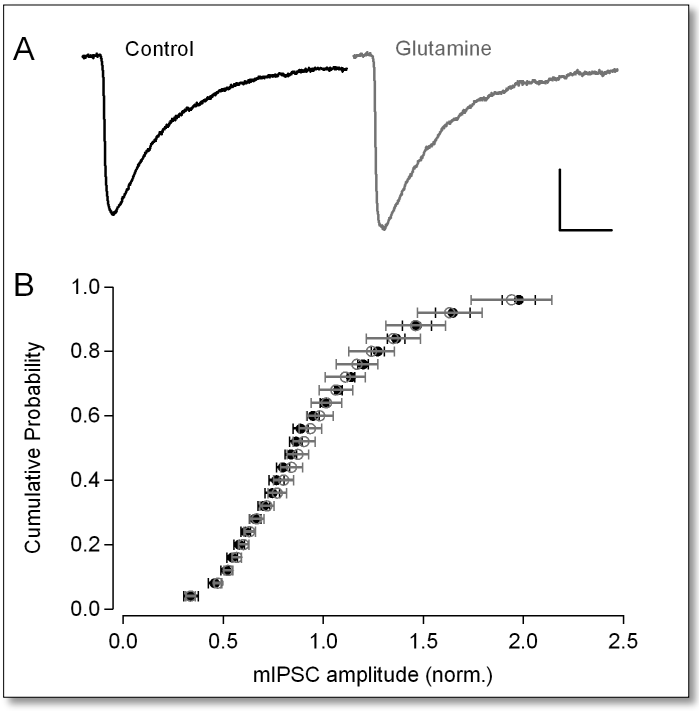

Figure 11. Exogenous glutamine has no effect on mIPSC amplitudes
A, Average mIPSC waveforms from a representative cell in control solution (black) and 500 μM glutamine (gray). Scale bars represent 10 pA and 20 ms. **B**, Cumulative probability histograms of normalized mIPSC amplitudes in control solution (black symbols) and after addition of glutamine (gray symbols). Results with 500 mM or 2 mM glutamine were no different and were pooled together. Data were averaged from 5 cells and error bars indicate SEM.

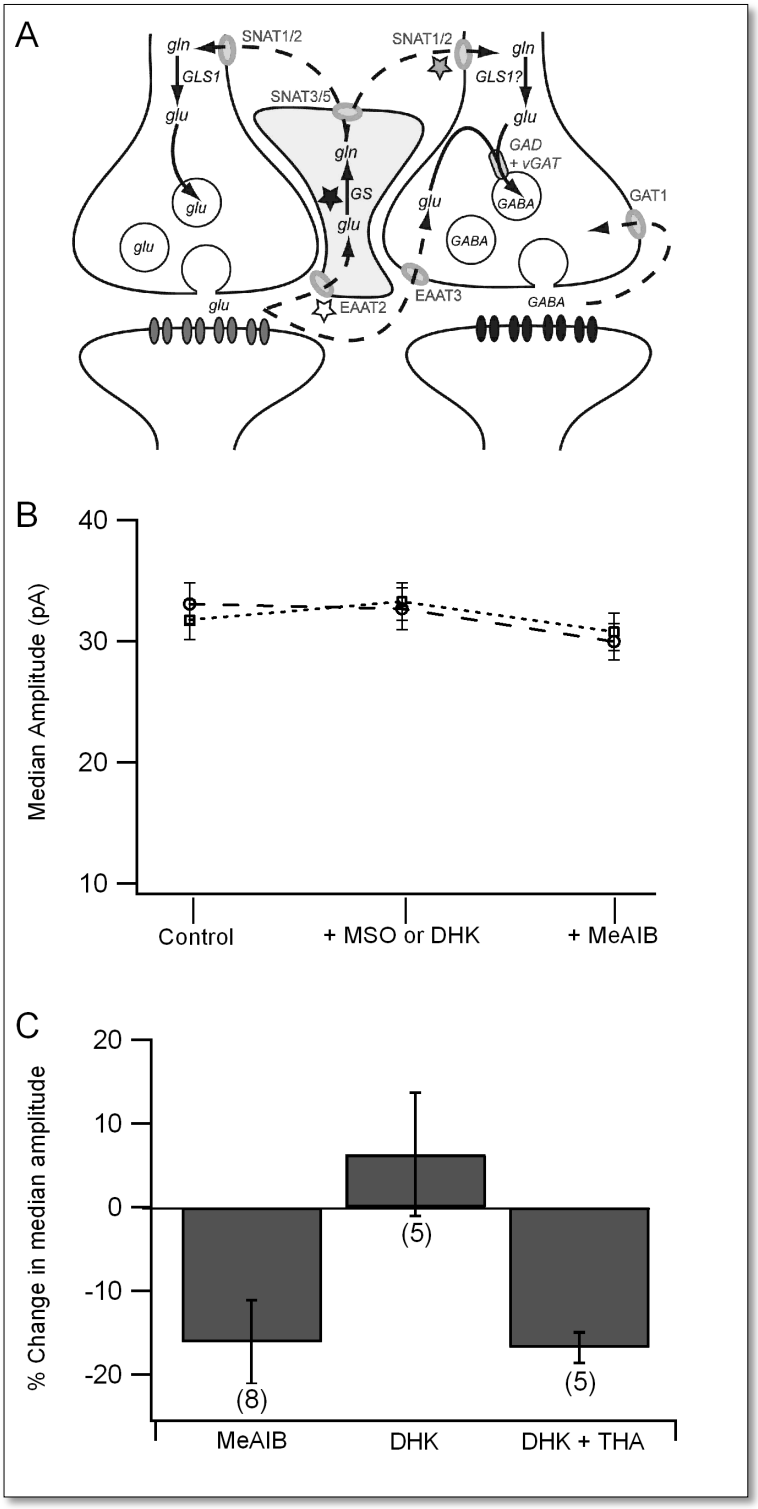

Figure 12. Astrocytes provide glutamine for GABA synthesis

Figure 12. Astrocytes provide glutamine for GABA synthesis
A, Schematic model of the metabolic pathways regulating the synaptic pool of GABA. Astrocytes (shaded) take up synaptically-released glutamate (*glu*), synthesize glutamine (*gln*), and export *gln* for uptake by both excitatory and inhibitory neurons using SNAT1/2. *Glu* for GABA synthesis can be converted from *gln* or can be taken up directly by EAAT3. Stars indicate the sites of action of DHK (white), MSO (black) and MeAIB (gray). THA non-selectively inhibits EAAT2 and 3. Abbreviations: GS, glutamine synthetase; GLS1, glutaminase type 1; GAD, glutamic acid decarboxylase; vGAT, vesicular GABA transporter; GAT1, GABA transporter 1; EAAT2 and 3, excitatory amino acid transporters 2 and 3. **B,** Lack of effect of addition of MeAIB during application of MSO (5 mM, circles, dashed line) or DHK (300 mM, squares, dotted line) on median mIPSC amplitudes. Error bars indicate SEM. **C**, Summary of the effects of MeAIB, DHK, and the combination of DHK and THA. Inhibition of EAAT2 alone with DHK does not affect mIPSC amplitudes, but additional inhibition of EAAT3 by THA produces a similar reduction compared with MeAIB. Number of cells indicated in parentheses. Error bars indicate SEM.

Discussion

System A transporters provide glutamine for synaptic GABA synthesis

Our results demonstrate that System A transporters on inhibitory neurons provide glutamine for neurotransmitter GABA synthesis. Pharmacological inhibition of these neuronal glutamine transporters with the selective inhibitor MeAIB reduced evoked IPSC amplitudes, and, correspondingly, reversibly reduced mIPSC amplitudes in hippocampal slices. We considered the possibility of a post-synaptic mechanism (i.e. a change in post-synaptic sensitivity to GABA), but no effect of MeAIB on currents elicited by exogenously applied GABA was observed. Moreover, the effect of MeAIB was abolished in the presence of agents that interrupted astrocytic glutamine synthesis, which would not be predicted if MeAIB acted on post-synaptic targets. Because of the relatively high concentration of MeAIB required to inhibit glutamine uptake, we were concerned with the possibility of non-specific effects. However, a similar reduction in mIPSC amplitudes was observed using histidine, an amino acid that competes with glutamine at multiple neutral amino acid transporters, suggesting that glutamine transporters were in fact the target of MeAIB. We also considered the possibility that the observed reduction in mIPSC amplitudes was the consequence of a pre-synaptic mechanism of action: either a reduction in vesicular GABA content or a decrease in the probability of vesicle release. To address the latter possibility we examined the effect of MeAIB on short-term synaptic plasticity, i.e. paired pulse ratios, which are interpreted to indicate changes in probability of release rather than in vesicular content. In agreement with the lack of change in paired-pulse ratio in the presence of MeAIB, we also observed no change in mIPSC

frequency, another common indicator of release probability. We therefore conclude, as in our earlier studies (Mathews & Diamond 2003), that the reductions in IPSC and mIPSC amplitude were due to an effect on GABA synthesis and, consequently, on vesicular content of GABA.

In a recent study (Liang et al. 2006), a role for glutamine supply in synaptic GABA release from hippocampal interneurons was manifest only after repetitive burst stimulation lasting 15 minutes. These results suggest that utilization of glutamine as a precursor for GABA synthesis occurs only when demand exceeds a resting level, although a threshold was not determined. In that study, MeAIB (5 mM) or MSO (1.5 mM) did not alter mIPSC amplitudes recorded in rat hippocampal CA1 pyramidal neurons. We consistently found an effect of MeAIB in the absence of neuronal activity (i.e. mIPSCs recorded in TTX; Figure 8), regardless of temperature. We also observed an effect of MeAIB on evoked IPSCs (Figure 7), which are recorded without blocking spontaneous synaptic activity, and the reduction in amplitudes was even greater than those seen in TTX (37% vs. 16% reduction, respectively). Therefore, we propose that the contribution of System A-mediated glutamine uptake to GABA synthesis does correlate with the level of synaptic activity, but that a significant role for this supply of precursor exists even under resting conditions (i.e. no synaptic activity). Perhaps our use of a higher (10 mM in our study vs. 5 mM) MeAIB concentration enabled us to detect these changes. There also may be other methodological differences, including age of animals (1-2 weeks in our study vs. 6 weeks). However, SNAT1 expression in rat hippocampal CA1 area is present as early as E17 and does not undergo obvious change with maturation (Weiss *et al.* 2003). Still, developmental changes in dependence on glutamine

supply are an interesting possibility to explore especially in light of evidence that expression of another substrate transporter, EAAT3, actually decreases during maturation (Furuta et al. 1997).

It is possible that MeAIB's effect on GABA synthesis was indirect, due to an effect on the uptake of glutamine and a subsequent reduction in glutamate release by excitatory neurons, thereby reducing glutamate that may be transferred between excitatory and inhibitory synapses (see Figure 12A). Glutamine is a source of neurotransmitter glutamate for excitatory neurons (Rothstein & Tabakoff 1984, Armano et al. 2002, Bacci et al. 2002), perhaps to a greater extent than for GABA (Szerb & O'Regan 1984). However, the effect of MeAIB on mIPSCs occurred rapidly (within 5 minutes) in the absence of neuronal activity (i.e. in TTX). Therefore it is unlikely that any effect on glutamate release from excitatory neurons would be sufficient to explain our results.

GABA vesicle content is dynamically regulated by supply of precursors

Our study is the first to demonstrate rapid changes in synaptic vesicle filling by disrupting the normally continuous glutamine supply from astrocytes to inhibitory neurons. In agreement with our conclusions, recent biochemical data indicate that GABA synthesis and vesicular filling are tightly coupled processes. First, the GAD isoform GAD65 is associated with synaptic vesicles where it forms complexes with the vesicular GABA transporter vGAT (Hsu *et al.* 2000). Second, newly synthesized GABA is packaged into vesicles preferentially over preformed GABA (Jin et al. 2003). Furthermore, unlike other neurotransmitter systems (eg. monoamines and glycine) that rely on reuptake to maintain neurotransmission, mice lacking the GABA reuptake

transporter GAT1 do not have impaired synaptic transmission (Jensen et al. 2003). Taken together with our results, these findings suggest that the GABA neurotransmitter system depends on synthesis more than reuptake. Due to the multiple metabolic pathways used by inhibitory neurons (see Figure 12A), the GABA neurotransmitter system has more potential sites of regulation than other systems. This study demonstrates that System A transporters, like EAAT3 (Mathews & Diamond 2003) and GAD (Engel *et al.* 2001), are key regulatory points at which inhibitory synaptic strength may be modulated by supply or metabolism of precursors.

Because spontaneous mIPSCs may represent a pool of vesicles separate from the readily releasable pool (Sara *et al.* 2005), we considered the possibility that we were measuring effects of MeAIB on a metabolically distinct subset of vesicles that did not participate in the rapidly recycling pool. Especially in light of a report that the requirement for glutamine in GABA synthesis is seen only after moderate stimulation (Liang et al. 2006), we speculated that glutamine may contribute only to a "reserve" pool of vesicles. Although the experiments depicted in Figure 6 show an effect of MeAIB on action potential-dependent release of GABA, we addressed this issue further by "evoking" mIPSCs with hyperosmolar solution in the presence of TTX, causing release of readily releasable vesicles. The amplitude reduction of these evoked mIPSC was reduced identical to that of the spontaneous pool, suggesting that the dependence on glutamine supply applies to all synaptic vesicles.

Glutamine is normally present at a high concentration (0.5 – 1 mM) in the cerebrospinal fluid (McGale et al. 1977) and *in vivo* microdialysis studies indicate that similar levels exist extracellularly in awake rat brains (Kanamori & Ross 2004). It is

likely that locally even higher concentrations exist, since astrocytic processes containing SNAT3, one of the transporters capable of mediating glutamine efflux (Figure 12A), ensheath GABAergic axon terminals (Boulland *et al.* 2002). Given the affinities of SNAT1 and 2 for glutamine [K_M = 0.5 and 1.6 mM, respectively; (Varoqui et al. 2000, Yao et al. 2000)], these transporters may operate at or near saturation under normal conditions. Addition of exogenous glutamine did not increase the GABA content of vesicles, supporting this conclusion. It is also possible that the enzyme that converts glutamine to glutamate, possibly GLS-1, is saturated rather than the transporters, and our experiments do not allow us to distinguish between these possibilities. It should be noted that immunohistochemical studies have demonstrated only rare localization of SNAT1 and SNAT2 to axon terminals in cortex (Conti & Melone 2006). Our results with MeAIB suggest that the location of System A transporters on inhibitory neurons is sufficiently near to synaptic terminals to rapidly influence neurotransmitter GABA metabolism.

In contrast to glutamine, glutamate applied exogenously, at concentrations higher than its normally very low extracellular level, increases the GABA content of vesicles (Mathews & Diamond 2003). Therefore, although System A transporters appear to be saturated, GAD has excess capacity allowing GABA synthesis to be up- as well as down-regulated by substrate supply. Extracellular levels of glutamate can bi-directionally regulate GABA vesicle content (Mathews & Diamond 2003), and local glutamate levels fluctuate with excitatory synaptic activity. Because extracellular glutamate levels are normally well below the K_M of EAAT3, we propose that glutamate uptake via EAAT3 is sensitive to excitatory activity, particularly in the hippocampal area CA1 where excitatory and inhibitory synapses lie in close proximity (Megias *et al.* 2001). In support

of this, in our previous work using protocols entailing relatively higher levels of synaptic activity (i.e. periodically evoked responses in the presence of strontium; (Mathews & Diamond 2003), blocking glial clearance of glutamate with DHK actually increased vesicle GABA content due to a relatively greater effect on extracellular glutamate that resulted in increased neuronal glutamate uptake. In this study, with synaptic activity blocked, DHK increased neuronal glutamate uptake sufficiently to counteract its effect on the glutamine pathway, as evidenced by our results with DHK in combination THA, resulting in no net change in vesicle content due to DHK alone.

We suggest that two independent substrate supply pathways exist for the synthesis of neurotransmitter GABA, mediated by SNAT1 or SNAT2 and EAAT3 (Figure 12A), and that both contribute to a dynamic equilibrium of synthesis and vesicular content. Their relative contributions are related both to the level of synaptic activity (and therefore glutamate release) and possibly to the intrinsic regulation of the transporters themselves. Changes in surface expression have been described for EAAT3 in response to activation of several signal transduction pathways (Fournier *et al.* 2004) and during LTP induction (Levenson *et al.* 2002), but similar regulation of SNATs in neurons remains to be explored. If System A transporters on inhibitory neurons are indeed saturated normally, then upregulation of SNAT expression is one potential mechanism by which enhancement of inhibition may be achieved in response to cellular signals.

CHAPTER III

ACTIVITY- AND AGE-DEPENDENT MODULATION OF GABAERGIC NEUROTRANSMISSION BY SNAT1-MEDIATED GLUTAMINE UPTAKE

Introduction

Normal brain function requires that inhibitory GABAergic systems adapt to a range of activity states, and increased demand for neurotransmitter GABA must be met with increased synthesis or seizures may result (Sepkuty *et al.* 2002, Kash *et al.* 1997). Because neurons are not capable of net production of glutamate, GABA's immediate metabolic precursor, interneurons rely on a supply of glutamate to maintain GABA synthesis and vesicular filling. Glutamine, which is cycled between astrocytes and neurons, can be converted to glutamate in neurons. Therefore, substrate transporters supplying either glutamate or glutamine may play a key role in linking activity-related demand for GABA to its rate of synthesis (see Figure 15A). The sodium-coupled neutral amino acid transporters SNAT1 and SNAT2 comprise "System A" that mediates most neuronal glutamine uptake (Broer & Brookes 2001), but whether either one plays an active role in shaping neurotransmission has not been determined.

Some studies suggest that System A-mediated glutamine uptake contributes to neurotransmitter pools only under conditions of increased metabolic demand (Tani *et al.* 2007, Bryant et al. 2009, Liang *et al.* 2006). However, inhibition of System A transporters rapidly reduces vesicular GABA content in immature hippocampal

interneurons under resting conditions (Fricke *et al.* 2007). Therefore, we investigated whether System A-mediated glutamine uptake can be regulated in order for interneurons to adapt to increased demand for GABA. Other elements involved in GABA synthesis (see Figure 15A) could also be regulated to influence inhibitory neurotransmission; however, because a concentration of glutamine capable of saturating System A transporters is available to neurons normally (McGale et al. 1977, Fricke et al. 2007), we hypothesized regulation of the surface activity of System A transporters would be an effective mechanism to modulate glutamine supply, GABA synthesis and vesicular GABA content. Moreover, because of the critical role of GABA signaling in coordinating excitatory synapse maturation and synaptogenesis (Wang & Kriegstein 2009, Ben-Ari et al. 2007), we hypothesized that the role of System A transporters at inhibitory synapses also may be developmentlally regulated.

Using electrophysiology in hippocampal slices in conjunction with protein expression studies and uptake assays in synaptosomes, we demonstrate that System A transporters serve both a constitutive and activity-dependent role in modulating vesicular GABA content and inhibitory synaptic strength. Synaptic depolarization up-regulates the surface activity of System A transporters and thus induces an increase in vesicular GABA content in both immature and mature hippocampus. However, because System A's basal uptake activity and therefore its constitutive contribution to vesicular GABA content diminishes over the first two postnatal weeks, its role in mature hippocampus is only manifest in an activity-dependent manner. Furthermore, our results support that these constitutive and activity-dependent roles are likely mediated by the SNAT1 subtype of System A transporters. Therefore, our results strongly support the hypothesis that the

surface activity of SNAT1, regulated both by depolarization and by developmental cues, is the key component in a novel mechanism to dynamically link metabolic demand for GABA with vesicular GABA content and inhibitory synaptic strength.

Experimental Procedures

Materials

α-(Methyl-amino) isobutyric acid (MeAIB), 3-Mercaptoproprionic acid (3-MPA), (1,2,5,6-Tetrahydropyridin-4-yl) methylphosphinic acid hydrate (TPMPA), 6,7-dinitroquinoxaline-2,3-dione (DNQX), CPG55845, and dizocilpine maleate (MK-801) were obtained from Sigma (St. Louis, MO, USA). Tetrodotoxin (TTX) was obtained from Tocris (Ellisville, MO, USA). [^{3}H]MeAIB was obtained from American Radiolabeled Chemicals. Jeffrey Erickson (LSU Health Sciences Center, New Orleans, LA) provided the rabbit polyclonal anti-SNAT1 (1:14,000) and anti-SNAT2 (1:1,000) antibodies used for western blot analysis and both antibodies have been characterized previously for specificity in rat brain (Varoqui *et al.* 2000, Yao *et al.* 2000). Additional antibodies used for western blot analysis include rabbit anti-EAAC1, 1:1000 (Alpha Diagnostic International); rabbit anti-GAT-1, 1:200 (Chemicon); mouse anti-GAD65, 1:500 (Chemicon); mouse anti-actin, 1:800,000 (Chemicon).

Preparation of hippocampal slices and patch clamp recording

Sprague Dawley rats at various ages were deeply anesthetized with isoflurane and decapitated under a protocol approved by Vanderbilt University's Institutional Animal Care and Use Committee. Hippocampi were removed and cut into 400μm sections using a vibratome (Vibratome Company, St. Louis, MO, USA) in an ice-cold oxygenated solution containing (in mmol/L): sucrose 75, NaCl 87, KCl 2.5, $MgCl_2$ 7, $CaCl_2$ 0.5, NaH_2PO_4 1.25, glucose 25, and $NaHCO_3$ 25. Slices were transferred to a chamber

containing artificial cerebrospinal fluid (ACSF), which contained (in mmol/L): NaCl 119, KCl 2.5, $MgCl_2$ 1.3, $CaCl_2$ 2.5, NaH_2PO_4 1, glucose 11, and $NaHCO_3$ 26.2, (pH 7.4, 290mOsm) and bubbled with 95% O_2/5% CO_2. Slices were incubated at 35°C for 30 min, then at room temperature (22-25°C) for 30 min before recording.

Patch pipettes with resistances of 3-5MΩ were filled with a solution containing (in mmol/L): cesium chloride 130, HEPES 10, EGTA 10, lidocaine *N*-ethyl bromide (QX-314) 1, Na^{2+}-GTP 0.2, and Mg^{2+}-ATP 2 (pH 7.4, 290 mOsm). For studies investigating the effects of high K^+-induced depolarization, KCl replaced cesium chloride and QX-314 was witheld from the solution. Whole cell recordings were obtained at a holding potential of -60mV with a MultiClamp 700A amplifier (Molecular Devices Corporation, Sunnyvale, CA, USA). Access resistance was monitored throughout the experiment and data were discarded if increase was more than 20%. Slices were perfused constantly at approximately 2 mL/min with ACSF (bubbled with 95% O_2/5% CO_2). Miniature inhibitory postsynaptic currents (mIPSCs) were recorded by adding TTX (0.5µmol/L), DNQX (10 µmol/L), and MK-801 (5µmol/L) to the recording solution. All experiments were performed at 22-25°C unless otherwise indicated.

After control currents were obtained, the effects of MeAIB, TPMPA or glutamine were evaluated after a 5 minute wash-in period. For TPMPA studies, CPG55845 (300nmol/L), a $GABA_B$ receptor antagonist, was included in the perfusate to block activity of TPMPA at $GABA_B$ receptors. For studies in which glutamic acid decarboxylase was inhibited, slices were incubated for 1h in 3-MPA (1mmol/L), then transferred to the recording chamber where the perfusate contained 250µmol/L 3-MPA. For experiments evaluating the effect of hyperexcitable state, slices were exposed to

2.5mmol/L KCl-ACSF (sham) or 10mmol/L KCl-ACSF (high K^+) for 20 minutes at room temperature and then transferred to the recording chamber where the remainder of the experiment was performed at 34°C. Slices were perfused with their respective ACSF for 10-20 minutes while a suitable patch clamp recording was obtained and when the whole-cell configuration was achieved, the level of neuronal activity was monitored in current clamp for an additional 5 minutes (eg. Figure 14A). After this period, the perfusate for all slices was replaced with 2.5mmol/L KCl-ACSF containing TTX, DNQX, and MK-801. Therefore, all recorded neurons had a period of at least 5 minutes after whole-cell recording configuration was achieved to allow equilibration of the intracellular and pipette solution contents prior to recording mIPSCs which would be sufficient to exclude potential variations in intracellular chloride concentration between neurons in sham and high K^+ treated slices that could have confounded our results. For studies examining the effect of TPMPA or MeAIB on mIPSCs after sham treatment or high K^+-induced depolarization, we recorded mIPSCs at one-minute, washed-in TPMPA or MeAIB for 5 minutes, and assessed the drug effects on mIPSCs recorded at ten minutes post-depolarization (see Figure 14B).

Immunohistochemistry

Postnatal day 8 and 25 Sprague-Dawley rats were deeply anesthetized with sodium pentobarbital. Transcardiac perfusion with 0.9% saline followed by a solution of 4% paraformaldehyde (freshly prepared, pH 7.2) was performed. Brains were post-fixed overnight at 4°C. Coronal sections (40 μm) were cut using a freezing microtome (Leica Microsystems, Germany). A mouse monoclonal anti-SNAT1 (N104/37) antibody was

used for immunohistochemistry (NeuroMabs, UC Davis). Both N104/37 and the rabbit polyclonal anti-SNAT1 antibodies recognized a 52kD band in immunoblots and their binding was blocked by preadsorption with the control peptide (data not shown). In addition, both SNAT1 antibodies displayed the same staining pattern in immature and mature hippocampus - a population of non-pyramidal neurons without appreciable staining of pyramidal neurons - although the rabbit polyclonal antibody demonstrated higher background staining (Figure 13). Immunohistochemistry was performed with free-floating sections blocked in 4% milk then incubated for 72 hours at 4°C in primary antibody. An optimal antibody dilution of 1:1000 was determined by evaluation of immunostaining over a range of dilutions. For secondary detection, sections were incubated in biotinylated anti-rabbit IgG (1:1000, Jackson Immunoresearch, West Grove, PA) for one hour at room temperature, then with avidin–biotin amplification reagent with horseradish peroxidase (Vectastain Elite ABC, Vector Laboratories, Burlingame, CA). Detection of horseradish peroxidase activity was achieved with the 3,3'-diaminobenzidine reaction.

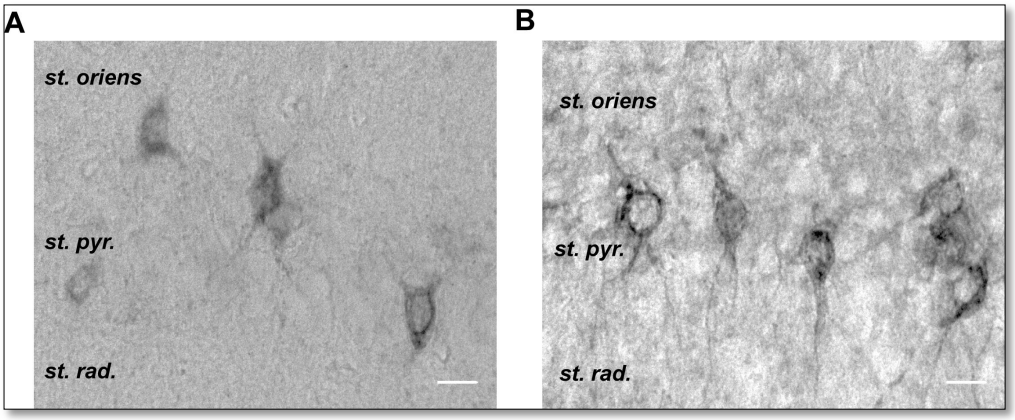

Figure 13. Rabbit polyclonal anti-SNAT1 and Mouse monoclonal anti-SNAT1 antibodies stain similar populations of non-pyramidal neurons in area CA1 of hippocampus

A, B, Both the mouse monocolonal anti-SNAT1 antibody (A) and the rabbit polyclonal anti-SNAT1 antibody (B) label a similar population of non-pyramidal neurons in area CA1 of rat hippocampus (P22) under identical conditions. The rabbit polyclonal anti-SNAT1 antibody demonstrated higher background staining. Scale bar represents 20μm.

Western Blot Analysis

Hippocampi were dissected from Sprague-Dawley rats at the postnatal day indicated, immediately frozen by dry ice, weighed, and stored at -80°C. Frozen tissue was placed in ice-cold solution (15μL/mg) containing 2% SDS and a protease inhibitor cocktail (1:1000, Sigma) and sonicated (Sonics Vibra Cell, Sonics & Materials, Inc., Newtown, CT). Protein concentrations were determined using the colorimetric *DC* Protein Assay (Bio-Rad). Samples were diluted 1:1 in 2X sample buffer (0.125M Tris base, 10% SDS, 10% glycerol, 0.02% Bromophenol blue, 4% β-mercaptoethanol, pH 6.8) and stored at -80°C. Equal amounts of protein (20μg for GAT-1 experiments, 10μg for all remaining experiments) were loaded on a 10% Tris-Glycine polyacrylamide gel. After electrophoretic separation, proteins were transferred to a PVDF membrane. Membranes were blocked with 5% milk plus 0.1% Tween 20 for 1 hour at room temperature, and incubated with the respective primary antibody overnight at 4°C. After several washes in PBS plus 0.1% Tween 20, membranes were incubated in horseradish peroxidase-conjugated secondary antibodies (1:20,000, Jackson Laboratories, Inc.) for 1 hour at room temperature. After several washes in PBS plus 0.1% Tween 20, membranes were developed with enhanced chemiluminescence reagents (ECL kit, Amersham) and then exposed to autoradiography film (Denville Scientific, Inc.). Measurements of each protein were conducted in the same sample, and all experiments were repeated in three different animals at each age.

Preparation of synaptosomes and [^{3}H]MeAIB uptake assays

Crude synaptosomes were prepared by dissecting hippocampi from immature (P8-11) and mature (P21-28) Sprague-Dawley rats which were immediately immersed in 3mL of an ice-cold solution containing (in mmol/L): sucrose 320, NaCl 119, KCl 2.5, $MgCl_2$ 1.3, $CaCl_2$ 2.5, NaH_2PO_4 1, glucose 11, and HEPES 10. Tissue was homogenized at 400 rpm using a Teflon-glass tissue homogenizer (Wheaton Instruments, Millville, NJ) followed by centrifugation at 2000 rpm for 10 minutes at 4°C. The supernatant was transferred to clean centrifugation tubes and centrifuged at 13,000 rpm for 15 min at 4°C. After discarding the supernatant, the pellet was re-suspended in 1 mL ACSF (10mM HEPES replacing $NaHCO_3$) and analyzed for protein concentration (Bio-Rad protein assay reagent, 1:5). For western blot analysis, synaptosomes were diluted 1:1 in sample buffer and probed for SNAT1, GAD65, and actin according to conditions specificed above. For uptake assays, samples containing 325µg protein each were incubated for 10 minutes (unless otherwise indicated) in a shaking water bath at 37°C prior to the addition of [^{3}H]MeAIB (167nmol/L; specific activity, 60Ci/mmol). After a 2.5 minute incubation, [^{3}H]MeAIB uptake was terminated by adding 1 mL ice-cold ACSF (choline chloride replacing sodium chloride). Uptake after high K^+-induced depolarization was examined according to the protocol published in (Ferguson *et al.* 2003). Samples were incubated in sham (2.5mmol/L KCl) or high potassium (10mmol/L KCl) ACSF for 15 minutes in a shaking water bath at 37°C. Samples were then immediately centrifuged at 13,000rpm for 2 minutes, their supernatants were discarded, and the pellets were resuspended with sham ACSF. All samples incubated in a shaking 37°C water bath for either 1 or 10 minutes prior to the addition of 167nmol/L [^{3}H]MeAIB for 2.5 minutes. Samples were

collected onto 0.3% polyethylenimine-coated glass fiber filters and washed three times with ice-cold ACSF using a Brandel cell harvester. Filters were immersed in scintillation fluid overnight and radioactivity was counted by scintillation spectrometry. All uptake assays were performed in triplicate and specific MeAIB uptake was determined by subtracting uptake in parallel samples prepared in ACSF where choline chloride replaced sodium chloride.

Data analysis

Electrophysiology

Individual mIPSCs meeting threshold criteria were detected using MiniAnalysis software (Synaptosoft, Fort Lee, NJ, USA) and visually inspected to exclude artifacts. Those single events arising from a stable baseline were used for analysis. At least 100 events were recorded from each cell in each condition, and the median was calculated. Each experiment was performed in multiple cells ('n') prepared from at least three animals. Assessment of pharmacological or high K^+ effects are reported as either a percent change from baseline ± SEM, a percent control of baseline ± SEM or a mean of the median mIPSC amplitude ± SD. Decay time constants were calculated for the mean of the median mIPSC amplitude (pClamp, Molecular Devices) and reported as either a single or weighted tau (F-test performed to assess best fit). Data evaluating drug or high K^+ effects were compared using a student's paired t-test unless otherwise indicated. Correlations between mIPSCs with time (Table 2) were assessed using Spearman's Rank Order Correlation Test.

Western Blot Analysis

Band intensity was measured with ImageJ software and normalized to the actin band for each protein. To compare the relative expression of SNAT1, EAAT3, and GAT-1 across development, we determined the ratio of each protein's level to that for GAD65 (also normalized to actin) to account for the formation and maturation of GABAergic synapses occurring during this time. Correlations between SNAT1 or SNAT2 protein expression with time were assessed using Spearman's Rank Order Correlation Test and developmental differences between the SNAT1:GAD65 protein ratio in synaptosomes were assessed using a student's t-test.

[^{3}H]MeAIB uptake assays

MeAIB uptake following high K^+-induced depolarization was normalized to the respective sham control and reported as a percent of control ± SEM. Statistical significance for both [^{3}H]MeAIB uptake experiments was assessed using a student's t-test.

Results

System A-dependent increase in quantal size after hyperexcitable state due to increased vesicular GABA content

Recent studies have demonstrated that neurons rely on glutamine supplied by System A transporters to sustain their neurotransmitter pools during periods of increased metabolic demand (Bryant et al. 2009, Liang et al. 2006, Tani et al. 2007). Because extracellular glutamine concentration is sufficient to saturate System A transporters, glutamine uptake is likely to be a key regulatory point within the glutamate-glutamine-GABA (glu-gln-GABA) pathway (see Figure 15A) capable of dynamically modulating vesicular GABA content. Consequently, we hypothesized that the glutamine supply to inhibitory synapses could be regulated through the surface activity of System A transporters. Evidence that individual synaptic vesicles do not contain enough GABA to saturate postsynaptic receptors in hippocampus has been provided by studies showing that the amplitude of mIPSCs directly varies within a certain range of GABA concentrations within the synaptic cleft ($[GABA]_{cleft}$), correlating with changes in vesicular GABA content (Mathews & Diamond 2003, Stafford et al. 2009, Hartmann et al. 2008).

We induced a hyperexcitable state in hippocampal slices by transiently elevating extracellular potassium, and we assessed quantal size immediately afterwards by recording mIPSCs in CA1 pyramidal neurons. Slices that were placed in ACSF containing 10mmol/L KCl (hereby termed high K^+-induced depolarization) demonstrated hyperexcitability that was characterized by a depolarization of the membrane potential by

approximately 15mV as well as an increase in the frequency of both action potentials and synaptic potentials recorded in the CA1 pyramidal neuron (Figure 14A). Sham treated slices were exposed to the identical protocol except control ACSF containing 2.5mmol/L KCl was substituted for the high K^+ ACSF. Following the 30 minute incubation, tetrodotoxin was added to the recording solution (control ACSF) and mIPSCs were recorded after allowing sufficient time for equilibration of the patch pipette solution with the intracellular contents (Figure 14B). In slices from immature (P8-12) rats, mIPSC amplitudes increased from 34 ± 9 pA immediately after high K^+-induced depolarization to 41 ± 12 pA ten minutes after depolarization ($p<0.05$, n=7; Figure 14Ci). This increase is reflected in a shift in the distribution of mIPSC amplitudes toward larger amplitudes at ten minutes post-depolarization ($p<0.01$, KS test; Figure 14Cii). The increase in quantal size was due to the high K^+ as no change in mIPSC amplitudes was observed following sham treatment ($p=0.3$, n=4; data not shown). Similarly, in mature (P21-28) slices, mIPSC amplitudes were increased from 32 ± 6 pA to 39 ± 7 pA ten minutes after high K^+-induced depolarization ($p<0.05$, n=7; Figure 14Di) and the amplitude distribution was shifted toward larger values ($p<0.01$, KS test; Figure 14Dii). No change in mIPSC amplitudes was observed following sham treatment ($p=0.9$, n=3; data not shown).

Since an increase in quantal size may result from either presynaptic or postsynaptic changes, we sought to distinguish between a change in synaptic $[GABA]_{cleft}$ and a change in post-synaptic receptor number or sensitivity by examining the effect of TPMPA on mIPSC amplitudes following high K^+-induced depolarization. TPMPA is a weak competitive antagonist at $GABA_A$ receptors characterized by a dissociation time constant that is more rapid than the syaptic cleft transient of GABA (Jones *et al.* 2001).

As a result, TPMPA's efficacy is very sensitive to synaptic $[GABA]_{cleft}$ and therefore to changes in vesicular GABA content (Liang et al. 2006, Hartmann et al. 2008). We first determined the concentration of TPMPA required to reduce mIPSC amplitudes by the same amount at each age following sham treatment. There was a slight difference in the concentration needed to reduce mIPSCs by 20-25% (Immature: 100µmol/L, 21 ± 4%, n=5; Mature: 85µmol/L, 26 ± 3%, n=5; Figure 14E), suggesting the baseline synaptic $[GABA]_{cleft}$ is dependent of developmental age. If the increase in quantal size following high K^+-induced depolarization were due to an elevation in synaptic $[GABA]_{cleft}$, TPMPA's efficacy should be proportionally reduced. In both immature and mature slices, the effect of TPMPA on mIPSC amplitudes after high K^+-induced depolarization was reduced compared with its effect in sham treatment (Immature: +4 ± 5%, $p<0.01$, n=5; Mature: -4 ± 8%, $p<0.05$, n=5; student's t-test, Figure 14E), consistent with augmented vesicle content and synaptic $[GABA]_{cleft}$ producing the observed increase in quantal size.

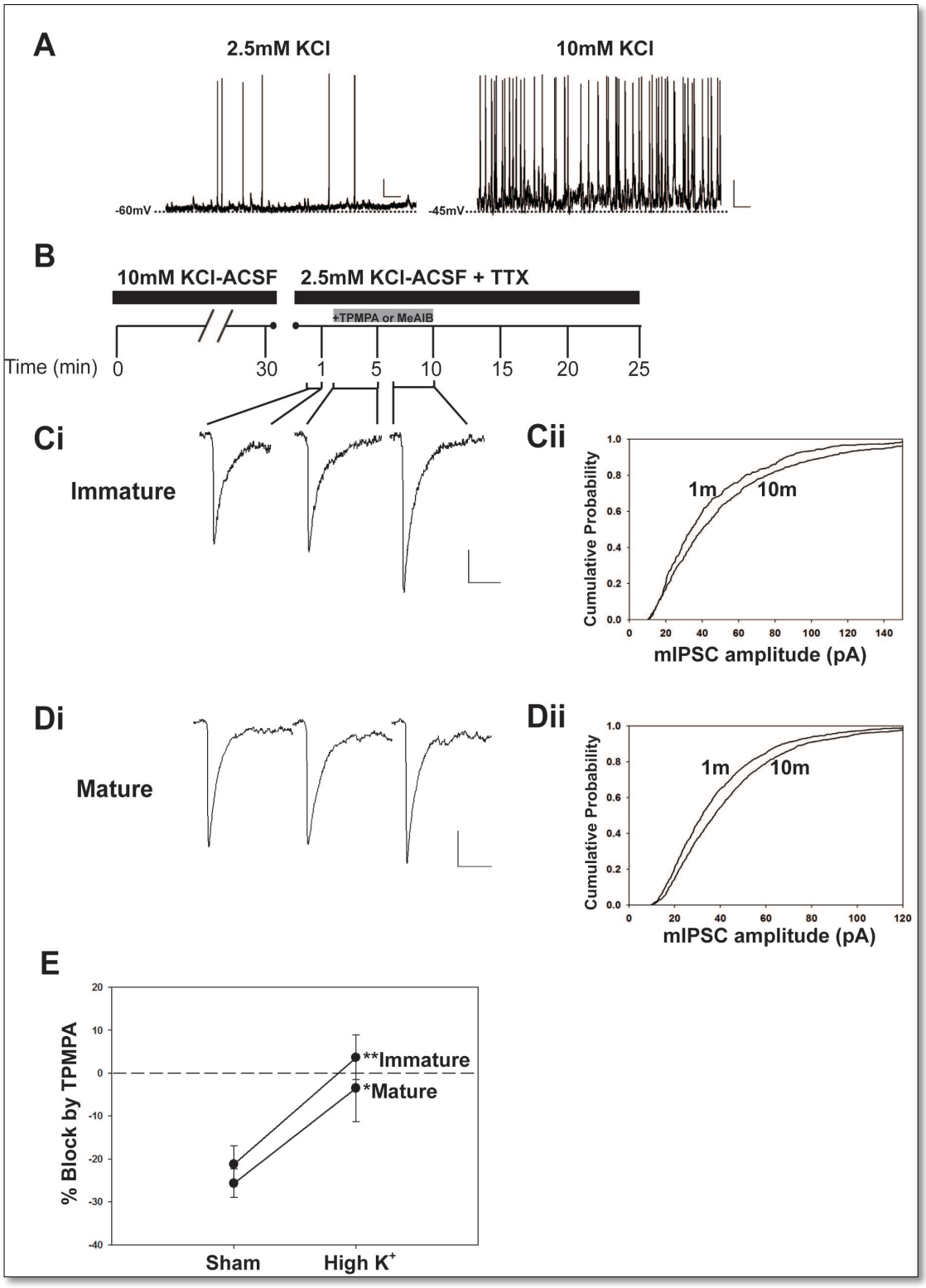

Figure 14. Synaptic depolarization augments quantal size due to increased vesicular GABA content

Figure 14. Synaptic depolarization augments quantal size due to increased vesicular GABA content

A, Representative action potential and spontaneous postsynaptic potential waveforms recorded from an immature CA1 pyramidal neuron during sham-treatment (2.5mmol/L KCl) or high K^+-induced depolarization (10mmol/L KCl). Note the approximate 15mV depolarization in the resting membrane potential. Scale bars represent 10mV and 1s. **B,** Time line of experimental protocol for high K^+-induced depolarization of hippocampal slices. **Ci, Di,** Representative averaged mIPSC waveforms at 1, 5, and 10 minutes following high K^+-induced depolarization. Scale bars, 10pA and 20ms. **Cii, Dii,** Cumulative probability histograms (n=7) of mIPSC amplitudes measured at 1 and 10 minutes following high K^+-induced depolarization of slices from immature (C) and mature (D) rats. **E,** Comparison of the effect of TPMPA on mIPSC amplitudes reported as % block by TPMPA following sham-treatment or high K^+-induced depolarization. The % block was determined by calculating the % change in mIPSC amplitudes at ten minutes compared with mIPSC amplitudes recorded within the first minute following high K^+. See text for 'n'; Error bars, SEM.

In both immature and mature slices, the increase in vesicular GABA content correlates with a time-dependent increase in mIPSC amplitudes, which peaked at ten minutes following high K^+-induced depolarization followed by a return to baseline values (Immature: $p<0.01$, n=7; Mature:, $p<0.05$, n=7; Repeated Measures ANOVA, Figure 15B,C). Consistent with a specific correlation, we observed no change in mIPSC amplitudes at any time following sham treatment (Immature: p=0.6, n=4; Mature: p=1.0, n=3; Repeated Measures ANOVA, Figure 15B,C). In order to determine if the increase in vesicular GABA content required uptake of glutamine via System A transporters, we tested the effect of MeAIB, a specific inhibitor of System A transporters, on mIPSC amplitudes following high K^+-induced depolarization. With an apparent affinity of 0.5-1.1mmol/L (Mackenzie et al. 2003, Yao et al. 2000), MeAIB has been used at 5–50mmol/L to selectively inhibit System A transporters in a variety of *in vitro* studies (Bacci *et al.* 2002, Rae *et al.* 2003, Liang et al. 2006, Fricke et al. 2007). MeAIB (10mmol/L) blocked the increase in mIPSC amplitudes following high K^+-induced depolarization in both immature (p=0.2, n=5; Figure 15B) and mature (p=0.9, n=5; Figure 15C) slices. These results suggest that the increase in vesicular GABA content required an ongoing supply of glutamine via System A transporters after the hyperexcitable period had ended.

A glutamine-dependent increase in synaptic GABA during periods of depolarization and increased neuronal activity could be mediated by an up-regulation of multiple elements within the glu-gln-GABA pathway (Figure 15A). However, because extracellular glutamine concentrations are capable of saturating System A transporters [(Fricke et al. 2007); see also Figure 19 below], we hypothesized that synaptic

depolarization could increase the surface activity of System A transporters, thereby increasing glutamine uptake and supply, GABA synthesis and vesicular GABA content. We also hypothesized that regulation of the activity of the physiologically relevent pool of System A transporters could be observed directly in synaptosomal membranes. To test these hypotheses, we measured the uptake of [^{3}H]MeAIB after high K^+-induced depolarization compared with sham treatment (analogous conditions to the slice experiments) in synaptosomes prepared from both immature and mature hippocampus. High K^+-induced depolarization increased [^{3}H]MeAIB uptake to 183 ± 31% of control ($p<0.05$, n=7) at one minute and to 151 ± 34% ($p=0.2$, n=9) at ten minutes in immature synaptosomes (Figure 15D). In mature synaptosomes, [^{3}H]MeAIB uptake was increased at ten minutes after high K^+-induced depolarization to 206 ± 43% ($p<0.05$, n=5; Figure 15E). Therefore, depolarization of synaptic membranes increased System A uptake activity within a similar time frame observed for augmentation of mIPSC amplitudes in intact slices.

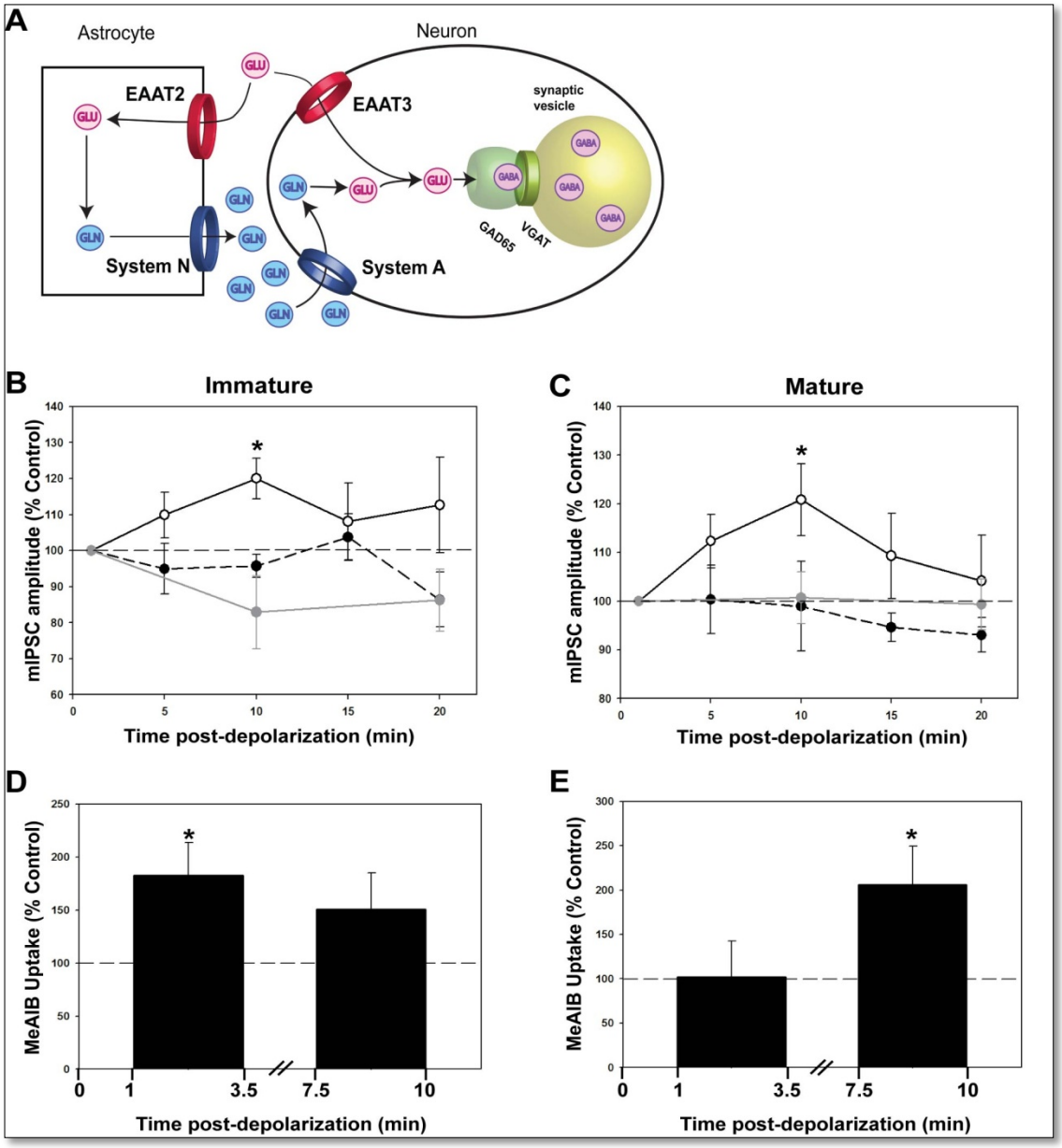

Figure 15. Synaptic depolarization augments System A surface activity to increase vesicular GABA content and inhibitory neurotransmission

Figure 15. Synaptic depolarization augments SNAT1 surface activity to increase vesicular GABA content and inhibitory neurotransmission

A, Schematic illustration of GABA biosynthetic pathways. GAD65 and the vesicular GABA transporter (vGAT) are part of a complex of proteins that link GABA synthesis and vesicular filling. Neurons acquire glutamate through direct uptake by EAAT3 or through the glutamate-glutamine-GABA pathway. In this pathway, astrocytes uptake glutamate via EAAT2, convert it to glutamine, and export glutamine via System N transporters for neuronal uptake by System A transporters. Neurons convert glutamine back to glutamate. **B, C,** Timecourse displaying the % of control mIPSC amplitudes across time following sham treatment (black circles), high K^+-induced depolarization (white circles) or high K^+ after 10mmol/L MeAIB (gray circles). The % of control mIPSC amplitude was calculated by normalizing the average median mIPSC amplitudes at each reported timepoint to the mIPSC amplitude at 1 minute. **D, E,** Comparison of [^{3}H]MeAIB uptake reported as % of control at 1 or 10 minutes after high K^+-induced depolarization. Bar width reflects incubation time of [^{3}H]MeAIB (2.5m). **A-D,** See text for 'n', *$p<0.05$; Error bars, SEM.

System A glutamine uptake contributes constitutively to synaptic GABA in an age-dependent manner

Thus far our results demonstrate that System A-mediated glutamine uptake is intrinsically regulated to rapidly augment GABA synthesis and quantal size after synaptic depolarization. Although the regulated role of System A did not differ between immature and mature hippocampus, we considered that the overall contribution of glutamine uptake to synaptic GABA might change during the course of developmental changes. In particular, we asked whether System A transporters constitutively supply glutamine for GABA synthesis, particularly early in development when GABA serves a major role in hippocampal synapse maturation and synaptogenesis, and whether System A's role changed during postnatal maturation. To address these questions, we examined the effect of MeAIB (10mmol/L) on mIPSC amplitudes in hippocampal slices at sequential postnatal ages. In immature slices, MeAIB rapidly reduced mIPSC amplitudes, consistent with System A transporters constitutively supplying glutamine for ongoing vesicular GABA synthesis (Figure 16). However, by the second postnatal week, the effect of MeAIB abruptly diminished and was absent in mature slices (Table 2, Figure 16). We concluded that the age-dependent change in MeAIB's effect on mIPSC amplitude ($p<0.05$) reflected a reduction in the constitutive contribution of glutamine uptake to vesicular GABA content and hypothesized that it could correlate with a change in the activity of System A transporters.

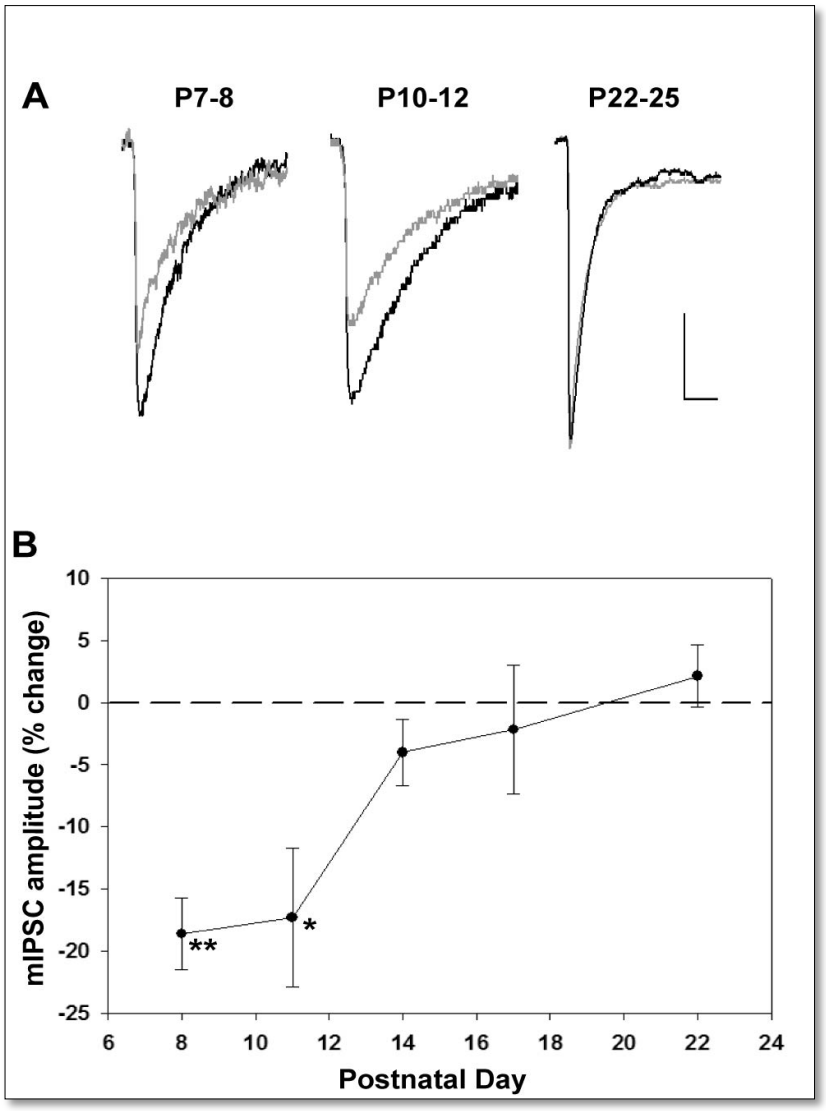

Figure 16. Age-dependent loss of the contribution of System A-mediated glutamine uptake to vesicular GABA content

A, Average mIPSC waveforms from a representative cell in control (black) and after 5 minutes in 10mM MeAIB at sequential postnatal ages. Scale bars, 10pA and 20ms. **B,** Plot demonstrating the age-dependent effect of MeAIB on mIPSC amplitudes. Each point represents the average percent change from baseline across a range of postnatal ages (n=7) (see Table 1). **p<0.01, *p<0.05; Error bars, SEM.

Table 2

Percent Change in mIPSC Characteristics in MeAIB			
Age	Amplitude	Decay	Frequency
P7-8 (n=7)	-19 ± 3**	+6 ± 6	+76 ± 28*
P10-12 (n=7)	-17 ± 6*	-6 ± 5	+48 ± 28
P14-15 (n=7)	-4 ± 3	+9 ± 5	+53 ± 19
P16-18 (n=7)	-2 ± 5	+20 ± 18	+19 ± 22
P22-25 (n=7)	+2 ± 3	+2 ± 5	+33 ± 8*
P42-44 (n=4)	+9 ± 6	+7 ± 13	+37 ± 53
Values are mean ± SEM. *p<0.01 and **p<0.001.			

We tested several alternative mechanisms that could explain the loss of MeAIB's effect on mIPSC amplitudes in mature slices. We considered that during maturation, System A-dependent glutamine uptake could become restricted to a sub-population of inhibitory synapses, and our statistical methods could fail to detect a change in mIPSC amplitudes in only a subpopulation of synapses. To address this possibility, we assumed that subpopulations of synapses have similar quantal sizes and thus we grouped mIPSCs into amplitude quartiles. We found that while MeAIB uniformly reduced mIPSC amplitudes in each quartile in immature slices (P7-8; $p<0.05$ in each quartile), mIPSC amplitudes were not reduced in any quartile in mature slices (data not shown). We also considered that if MeAIB reduced the probability of vesicle release or altered postsynaptic membrane properties, and these "non-specific" effects were somehow age-dependent, we should observe an effect of MeAIB on mIPSC frequency or mIPSC shape (eg. rate of decay) correlating with postnatal age. However, we did not observe a correlation between postnatal age and either mIPSC frequency ($p=0.5$) or mIPSC decay time constant ($p=0.8$) (Table 2). Therefore, the most likely conclusion is that the age-dependent reduction in System A's constitutive contribution of glutamine to synaptic GABA is due to a uniform loss of System A-dependent uptake at mature inhibitory synapses.

System A activity at inhibitory synapses mediates an age-dependent loss of glutamine's contribution to vesicular GABA

To test the hypothesis that there is an age-dependent loss in the constitutive surface activity of System A transporters, we assayed both the expression and the activity of System A transporters. We first asked where SNAT1 and SNAT2 protein were expressed and whether there was a change in their expression in hippocampal area CA1 during maturation. We found that SNAT1 was expressed selectively in a population of non-pyramidal neurons in area CA1, and that this pattern was indistinguishable between immature and mature hippocampus (Figure 17A). We did not observe a clear cell-specific pattern of SNAT2 expression (data not shown).

Next we measured total SNAT1 and SNAT2 protein in whole hippocampal lysates at sequential postnatal ages using a semiquantitative Western blot approach. SNAT1 protein was highly expressed in immature hippocampus, and gradually decreased throughout postnatal development to adult levels ($p<0.001$, $n=3$; Figure 17B), paralleling the age-dependent loss of System A's constitutive contribution of glutamine to synaptic GABA (Figure 15). In contrast to SNAT1, the immature hippocampus expressed a low amount of SNAT2 protein that increased to adult levels ($n=3$; Figure 17). Although we normalized our SNAT measurements to actin levels, because SNAT1 expression appeared restricted to interneurons, we considered the level of glutamic acid decarboxylase 65, (GAD65), the synaptic isoform of GAD, to be a more appropriate comparison for the physiological relevance of the age-dependent change in SNAT1 expression. In agreement with reports that the number of GABAergic synapses rapidly expands in hippocampus during the first three postnatal weeks (Danglot et al. 2006), we

found that GAD65 expression gradually increased (Figure 17B). When we calculated the ratio of SNAT1 levels to GAD65 as an indicator of its expression at relevant inhibitory synapses, SNAT1 levels actually decreased approximately 25-fold during postnatal maturation (Figure 17B). We also examined the expression profile of two additional transporters involved in synaptic GABA metabolism: the excitatory amino acid transporter, EAAT3 (see Figure 15A), and the GABA reuptake transporter, GAT-1, to determine if SNAT1 expression is uniquely regulated during development. The relative expression ratio for each transporter displayed a different temporal pattern (Figure 17D), supporting a highly specific age-dependent regulation of SNAT1 expression by inhibitory neurons.

These findings demonstrate that SNAT1 in the hippocampus is expressed selectively by interneurons and that the 25-fold decrease in amount of SNAT1 expressed at developing inhibitory synapses highly correlates with the age-dependent loss of System A's constitutive contribution of glutamine to synaptic GABA. SNAT2's developmental expression profile was contrary to the observed physiological effects. Therefore, our findings support that SNAT1, not SNAT2, likely functions as the transporter mediating glutamine uptake for synaptic GABA in hippocampus and thus we focused protein expression changes in subsequent studies on SNAT1.

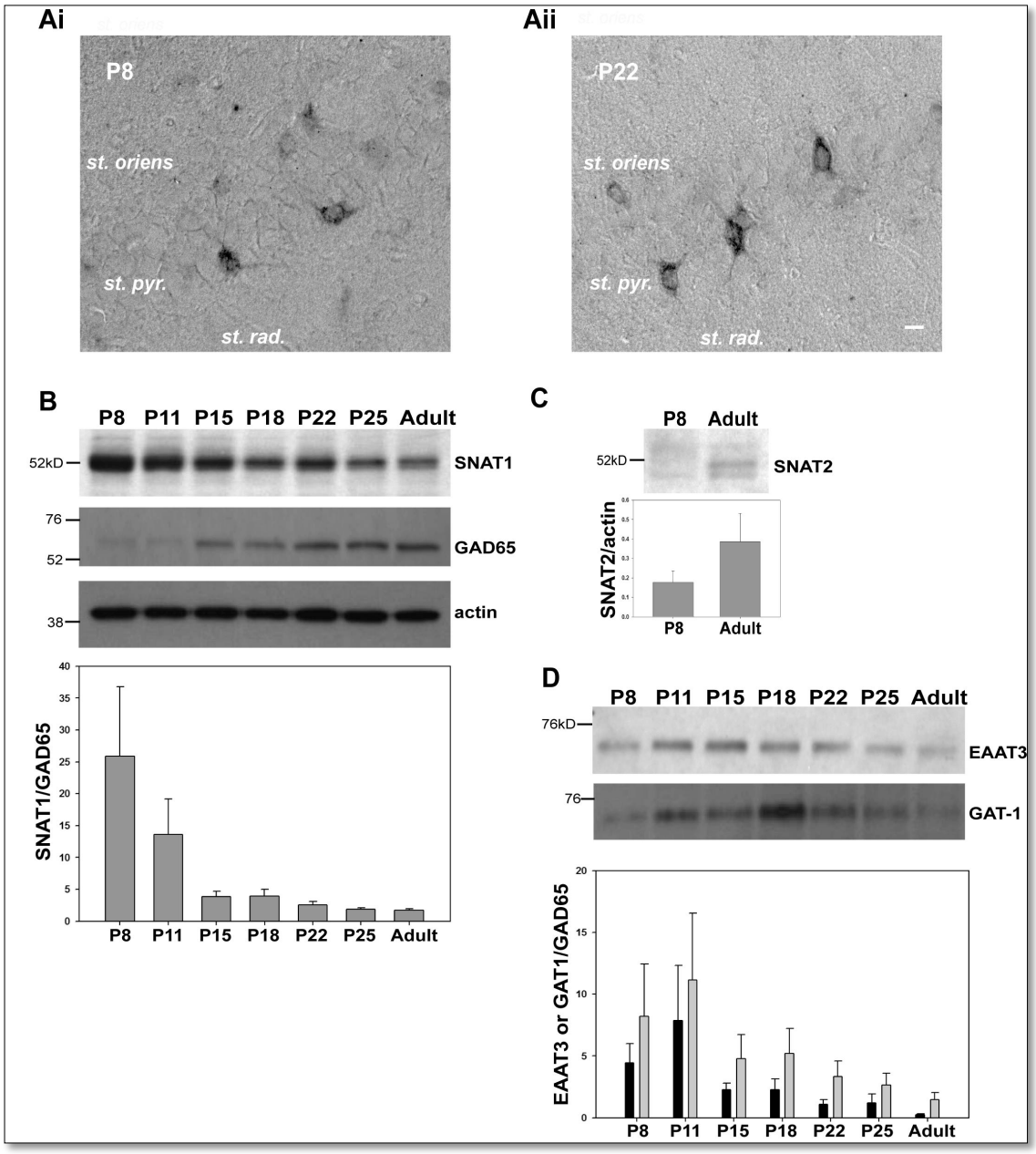

Figure 17. SNAT1 is selectively expressed by interneurons in hippocampal area CA1 and SNAT1 expression gradually decreases in an age-dependent manner

Figure 17. SNAT1 is selectively expressed by interneurons in hippocampal area CA1 and the SNAT1 expression gradually decreases in an age-dependent manner **Ai-ii**, Non-pyramidal cell bodies localized in *stratum oriens* (*st. oriens*), *st. radiatum* (*st. rad.*) and bordering *stratum pyramidale* (*st. pyr*.) in both immature (Ai) and mature (Aii) hippocampus positively express SNAT1. Scale bar represents 20μm. **B, D,** Representative western blots illustrating amount of SNAT1, GAD65, EAAT3, and GAT-1 expressed in whole hippocampal lysates from sequential postnatal ages and adulthood. Actin was used as a loading control. Bar graphs compare relative expression levels of the various transporters in panels B and D after normalization to GAD65 (n=3). **C,** Representative western blot illustrating amount of SNAT2 protein expressed in immature and adult hippocampus. Bar graph compares SNAT2 protein levels after normalization to actin (n=3). **B-D,** The position of protein standards is shown on the left. Error bars, SEM.

We hypothesized that the physiologically relevant pool of System A transporters would be enriched in the synaptosomal fraction, and that age-dependent changes in SNAT1 protein would be even more pronounced in this fraction than in whole lysates. Consistent with this hypothesis, immature synaptosomes expressed an increased amount of SNAT1 protein relative to mature synaptosomes ($p<0.01$, $n=3$; Figure 18A,B). In addition, the proportional difference in amount of SNAT1 protein (raw signal) expressed between immature and mature hippocampus was magnified in the synaptosomal fraction (2.7 ± 0.6 fold higher) compared with the whole lysate (1.8 ± 0.4 fold higher; data not shown). To further investigate whether SNAT1 basal uptake activity correlated with its protein levels, we measured uptake of [^{3}H]MeAIB in immature and mature hippocampal synaptosomes. After a 2.5 minute incubation, basal [^{3}H]MeAIB uptake was almost four-fold higher in immature compared with mature synaptosomes ($p<0.05$, $n=3$; Figure 18C) and this difference was conserved with both 5 and 10 minute incubations of [^{3}H]MeAIB (data not shown). Although MeAIB is a substrate for both SNAT1 and SNAT2, the developmental decrease in SNAT1 protein expression in synaptosomes is reflected in a parallel reduction in MeAIB uptake, and therefore interneuron terminals likely selectively reduce SNAT1 uptake activity in an age-dependent manner. Therefore, we conclude that SNAT1 protein expression and, even more so, its surface activity at inhibitory synapses is developmentally regulated and likely governs the age-dependence of System A's constitutive contribution to synaptic GABA.

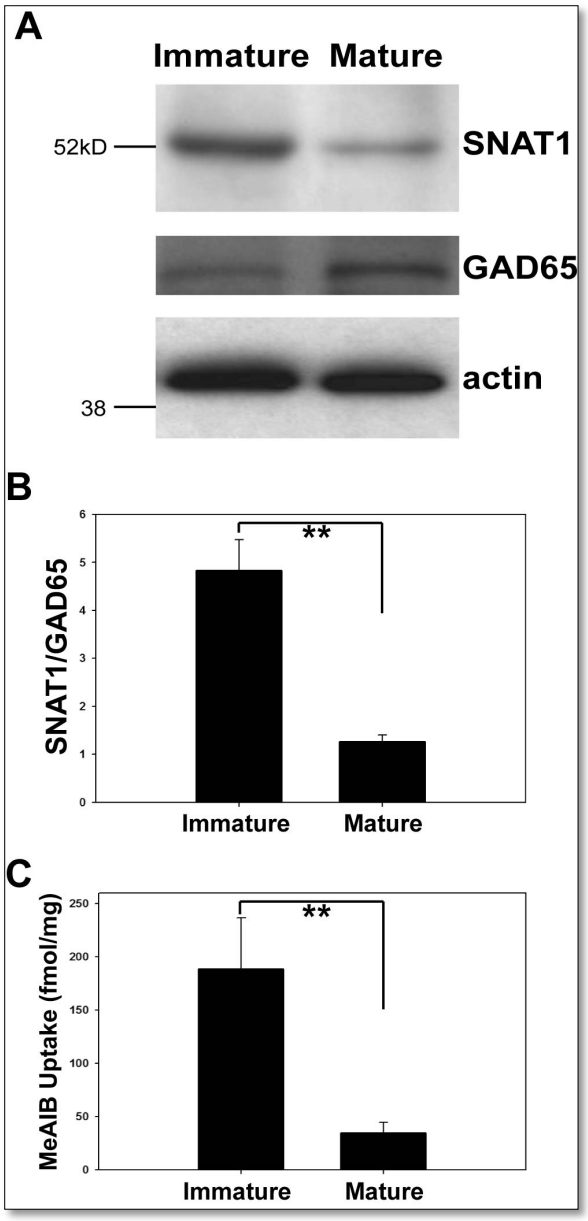

Figure 18. Reduced SNAT1 surface activity at mature inhibitory synapses mediates age-dependent loss of glutamine's constitutive contribution to synaptic GABA
A, Representative western blots illustrating amount of SNAT1 and GAD65 protein expressed in immature and mature hippocampal synaptosomes. The position of protein standards is shown on the left. Actin served as a loading control. **B,** Comparison of the relative amount of SNAT1 expressed in panel A after normalization to GAD65 levels (n=3), **p<0.01. **C,** Comparison of MeAIB uptake reported as fmol/mg protein between immature and mature hippocampal synaptosomes (n=3), *p<0.05. **B-C,** Error bars, SEM.

SNAT1 surface activity is the only age-dependent change governing the changing contribution of glutamine to vesicular GABA

Thus far our results support that System A transporters serve both a constitutive and activity-dependent role in modulating vesicular GABA content and inhibitory synaptic strength. These roles are governed by the uptake activity of System A transporters (although likely SNAT1, see Discussion), which are dynamically regulated by both developmental cues and synaptic depolarization. Although glutamine uptake appears to be the key regulatory point in the glu-gln-GABA pathway at synapses, we considered that developmental structural changes in the hippocampus, particularly in the relationships between inhibitory synapses and astrocytic processes or excitatory synapses could also result in changes in substrate (glutamine or glutamate, respectively) availability (see Figure 15A). To address this possibility, we examined if a decrease in glutamine supply upstream or a decrease in the contribution of new GABA synthesis to vesicular filling downstream of System A transporters could explain the age-dependent loss of System A's constitutive contribution of glutamine to synaptic GABA.

We first investigated potential changes in glutamine supply by examining the effect of exogenous L-glutamine on mIPSC amplitudes. We confirmed our previous report that addition of exogenous L-glutamine (2mmol/L) had no effect on mIPSC amplitudes in immature hippocampal slices, arguing that endogenous extracellular glutamine levels already saturate System A transporters ((Fricke et al. 2007), Figure 19A, B). Therefore, if endogenous extracellular glutamine were sufficiently reduced in mature hippocampus such that glutamine no longer constitutively contributed to synaptic GABA, then addition of exogenous L-glutamine should increase mIPSC amplitudes.

However, exogenous L-glutamine had no effect on mIPSC amplitudes in mature slices either (101 ± 5% of control, n=5, p=0.8; Figure 19A,B). Next, we investigated if mature synaptic vesicles rely less on newly synthesized GABA for vesicle filling by comparing the effect of the GAD inhibitor, 3-mercaptoproprionic acid (3-MPA), on mIPSC amplitudes from immature and mature slices. If newly synthesized GABA is not required for vesicle filling in mature neurons, inhibiting GAD should have no effect on mIPSC amplitudes. However, 3-MPA (1mmol/L) reduced mIPSC amplitudes to a similar extent in immature and mature slices (Immature: 35 ± 9 pA, n=10 vs. 25 ± 7 pA after 3-MPA, $p<0.05$, n=7; Mature: 36 ± 5 pA, n=18 vs. 30 ± 5 pA after 3-MPA, $p<0.05$, n=5; student's t-test, Figure 19C,D). These results support that a decrease in System A-mediated glutamine uptake, and not other elements of the glu-gln-GABA pathway, mediates the age-dependent loss of System A's constitutive contribution of glutamine to synaptic GABA.

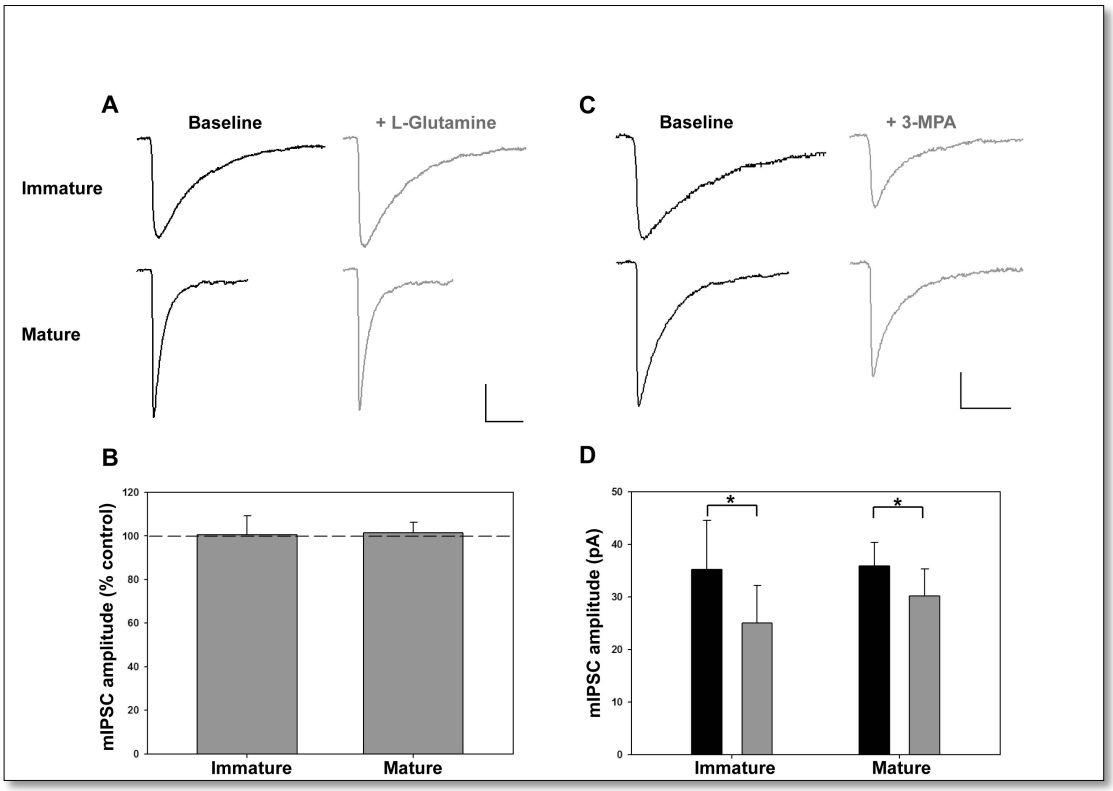

Figure 19. Developing inhibitory synapses do not down-regulate constitutive activity of the entire glu-gln-GABA cycle

A, Representative average mIPSC waveforms in control (black) and after 5 min. in exogenous 2mM glutamine from immature and mature slices. **B,** Comparison of the effect of 2mM exogenous glutamine reported as a percent control of baseline (n=5). Error bars, SEM. **C,** Average representative mIPSC waveforms from control (black) neurons and neurons exposed to 1mM 3-MPA in immature and mature slices. **D,** Comparison of mIPSC amplitudes in control (black) and in 1mM 3-MPA ACSF (see text for 'n') reported as average median mIPSC amplitudes. *$p<0.05$; Error bars, SD. **A, C,** Scale bars, 10pA and 20ms.

Discussion

Coupling between GABA synthesis and vesicle filling (Jin et al. 2003) provides a mechanism whereby supply of GABA's metabolic precursors to inhibitory synapses may dynamically up- or down-regulate vesicular GABA content and quantal size. Even though dynamic changes in vesicle content are a newly recognized form of synaptic plasticity (Edwards 2007), there are very few examples where this plasticity is observed under physiological conditions, and these examples have demonstrated that supply of glutamate by EAAT3 rapidly modulates inhibitory synaptic strength (Mathews & Diamond 2003, Stafford et al. 2009, Hartmann et al. 2008). We now have demonstrated that the surface activity of System A transporters, governing glutamine uptake at inhibitory synapses, is acutely regulated to modulate quantal size, providing the first example of such regulation of a substrate transporter dynamically shaping fast neurotransmission. We also have demonstrated that the constitutive contribution of System A-mediated glutamine uptake to synaptic GABA is determined by a developmentally regulated change in the surface activity of these transporters, paralleling a maturational change in the role of GABA in the hippocampus.

We showed previously that uptake of exogenous (Mathews & Diamond 2003) or synaptically released (Stafford et al. 2009) glutamate by EAAT3 enhances quantal release of synaptic GABA (see Figure 15A). However, because astrocytes maintain very low extracellular glutamate levels, this metabolic pathway may only contribute to synaptic GABA under conditions in which extracellular glutamate overwhelms astrocytic glutamate transporters. In contrast, extracellular glutamine exists at high levels and there

should be a continuous supply available to inhibitory synapses. Moreover, astrocytic processes expressing SNAT3 or SNAT5, the System N transporters capable of mediating glutamine efflux (Figure 15A), ensheath GABAergic axon terminals (Boulland et al. 2002, Cubelos *et al.* 2005) suggesting that glutamine may be locally maintained at higher levels than measured in the cerebrospinal fluid. Of the two independent substrate supply pathways (EAAT3 and System A), the System A pathway appears to be regulated intrinsically, by regulation of transporter activity rather than substrate availability, it is likely to play a unique role in homeostatic plasticity of inhibitory synapses.

SNAT1 is a key regulator of GABA metabolism at inhibitory synaptic terminals

Although both System A transporters, SNAT1 and SNAT2, have been viewed traditionally as potential suppliers of glutamine for synaptic glutamate and GABA (Chaudhry *et al.* 2002, Bak et al. 2006), recent immunohistochemical studies support that these two transporters have distinct and non-overlapping expression patterns in the hippocampus. SNAT2 is selectively expressed by excitatory neurons in adult hippocampus (Jenstad *et al.* 2009, Gonzalez-Gonzalez et al. 2005), and does not appear to play a significant role in neurotransmitter glutamate metabolism (Kam & Nicoll 2007, Grewal *et al.* 2009). In contrast, our immunohistochemical findings are supported by a recent study demonstrating SNAT1 protein expression limited to GAD67-expressing neurons in area CA1 (Solbu *et al.*). This study also demonstrated co-localization of SNAT1 protein with the vesicular GABA transporter (vGAT), a marker for GABAergic presynaptic terminals, at axon varicosities in cortical interneuron-enriched cultures. Moreover, in our study, SNAT2 protein actually increased with age (Figure 17), opposite

to the electrophysiological and biochemical studies showing a maturational loss in System A's constitutive contribution of glutamine to synaptic GABA (Figure 16). Although we did not rule out a role for SNAT2 in neurotransmitter GABA metabolism, we have provided multiple lines of evidence implicating SNAT1. Therefore, although the synaptic localization of SNAT1 has been controversial previously (Melone et al. 2004, Conti & Melone 2006), our current study and that of Solbu et al. strongly support the conclusion that SNAT1 functions at or near inhibitory synaptic terminals to supply glutamine for neurotransmitter synthesis.

Activity-dependent regulation of System A surface activity modulates vesicular GABA content

Our study is the first to demonstrate dynamic modulation of inhibitory synaptic strength via regulation of the surface activity of a presynaptic substrate transporter. Because our findings show that extracellular glutamine levels are sufficient to saturate System A transporters, altering glutamine supply to inhibitory synapses requires up- or down-regulation of their surface activity. Previous studies in mature animals were able to demonstrate a requirement for glutamine to maintain neurotransmitter pools only during periods of increased metabolic demand (Bryant et al. 2009, Liang et al. 2006, Tani et al. 2007). Whether specific elements involved in the cycling of glutamine between astrocytes and neurons can be up-regulated had not been previously explored. However, our results show that inhibitory neurons adapt to an increase in metabolic demand by increasing System A surface activity, offering a mechanism to mediate this increase in glutamine contribution to the GABA neurotransmitter pool. In fact, our results are

consistent with a previous study examining activity-dependent changes in glu-gln-GABA cycling (Liang et al. 2006) and may explain the underlying mechanism for these changes.

Several studies have demonstrated that hyperexcitability and acute seizure-like activity can be induced in hippocampal slices by elevating extracellular potassium (Dzhala & Staley 2003, Jensen & Yaari 1997, Traynelis & Dingledine 1988). We induced a more moderate level of hyperexcitability with the goal of depolarizing neurons, increasing synaptic activity and creating a higher demand for GABA. After 30 minutes, quantal size at GABAergic synapses was transiently increased. Interestingly, the increase in quantal size was delayed, appearing within 10 minutes after the depolarization protocol. There are two possible explanations for this finding: One explanation is that although System A surface activity is increased during the period of hyperexcitability, other factors explain why quantal size does not increase until after membrane potential or level of synaptic activity returns to normal. For example, System A activity could increase to maintain GABA synthesis and vesicular filling during the hyperexcitable state, compensating for increased demand. When synaptic activity is halted abruptly by addition of tetrodotoxin, the higher rate of GABA synthesis and slower rate of vesicular release may result in an "overfilling" of vesicles. Alternatively, elevated K^+ could impair the function of System A transporters due to reduction of the electrochemical gradient driving glutamine uptake such that an increase in glutamine uptake occurs only when a normal gradient is restored. The second explanation is that there is a delay in the upregulation in System A surface activity in response to depolarization. For example, it may take minutes for biochemical signals to translate into increased surface activity, although the precise mechanism of transporter regulation and the signals involved have

not been determined. Our synaptosomal uptake assays do not help differentiate between the two possible explanations for the delay. In the immature synaptosomes, System A function was increased immediately following depolarization, whereas in the mature synaptosomes, the increase was observed after a delay. Therefore, it remains a possibility that different mechanisms are involved in the activity-dependent role of System A transporters at different ages.

Although we have not determined the precise mechanism by which the surface activity of System A transporters is regulated, we have demonstrated that depolarization of synaptosomal membranes is sufficient to upregulate System A surface activity and therefore can account for the changes we observed in whole hippocampal slices. The mechanism may involve either regulation of System A's catalytic efficiency or translocation of transporters to the surface, or both. Dual modes of transporter regulation involving both surface expression and catalytic activity are common, as previous studies have identified independent mechanisms that regulate EAAT3 (Gonzalez *et al.* 2002), GLUT4 (Somwar *et al.* 2001), and NET (Apparsundaram *et al.* 2001) activity. Both the mechanism and the specific signaling molecules that regulate System A surface activity need to be resolved. Moreover, developmental differences in the regulation of System A transport activity may explain why its surface activity increases more rapidly in immature than mature synaptosomes after high K^+-induced depolarization (Figure 15C,D). We propose that the acute regulation of System A transport is an intrinsic homeostatic mechanism to link metabolic demand with substrate supply. Likewise, discovery of this important role of System A transporters suggests that future studies could explore its function in disorders characterized by chronic hyperexcitability such as epilepsy.

Implications of altered System A surface activity during hippocampal maturation

We considered alternative mechanisms that might explain the age-dependent loss in glutamine's constitutive contribution to synaptic GABA. We first considered that the degree of saturation of $GABA_A$ receptors might increase during development. For example, if the synaptic $[GABA]_{cleft}$ or the $GABA_A$ receptor affinity for GABA were increased during postnatal maturation, modest changes in vesicular GABA content might have little or no consequence on the inhibitory postsynaptic response. Several findings argue against this explanation. First, we demonstrated that similar concentrations of the low affinity antagonist TPMPA reduced mIPSC amplitudes equivalently in immature and mature slices (Figure 14E). In fact, slightly less TPMPA was required to block mature mIPSCs suggesting a lower degree of $GABA_A$ receptor saturation. Second, high K^+-induced depolarization increased synaptic $[GABA]_{cleft}$ and thus mIPSC amplitudes by a similar extent at both ages (Figures 14-15), indicating that a single vesicle does not saturate post-synaptic receptors at either age. Lastly, previous studies indicated that affinity of synaptic $GABA_A$ receptors is reduced during maturation (Laurie et al. 1992, Bohme *et al.* 2004). We did consider the additional explanation that glutamine's constitutive contribution to synaptic GABA at mature synapses is slower, requiring hours, not minutes, to observe changes, similar to observations in glutamatergic neurons (Kam & Nicoll 2007). If this were true, it still would suggest a developmental change in the metabolic pool of precursor for GABA synthesis that disconnects System A transporters from the rapidly and dynamically modulated neurotransmitter pool measured in our experiments. Therefore, we conclude that SNAT1 protein expression and consequentially its surface activity at inhibitory synapses is developmentally regulated

and directly mediates the age-dependent loss in System A's constitutive contribution of glutamine to synaptic GABA.

Why would developing inhibitory neurons scale down the constitutive surface activity of System A transporters but maintain an intrinsic mechanism to increase its surface activity upon synaptic depolarization? During early postnatal development, GABA coordinates excitatory synapse maturation and therefore early inhibitory synapses likely encounter little extracellular glutamate supply. We hypothesize System A's constitutive surface activity possibly functions to sustain synaptic GABA during this period and thus System A function may be critical during hippocampal maturation. After a sufficient density of excitatory synapses form, GABA metabolism may be additionally modulated by EAAT3-dependent glutamate uptake. Still, neurons maintain the intrinsic mechanism for System A transporters to modulate GABA synthesis during periods of increased excitability. In summary, our results demonstrate that regulation of System A surface activity is a novel mechanism linking metabolic demands for GABA to its vesicular packaging, adapting inhibitory neurotransmission to a range of activity states.

CHAPTER IV

CONCLUSIONS, DISCUSSION AND OUTSTANDING QUESTIONS

GABA functions as the primary inhibitory neurotransmitter in the mammalian brain. In hippocampus, GABA serves multiple roles during development and throughout adulthood, which include to: 1) orchestrate synapse maturation and synaptogenesis, 2) maintain inhibitory synaptic transmission, and 3) regulate the excitability and synchronize the neuronal output of principal neurons. Because normal brain function requires intact inhibitory synaptic transmission, interneurons must possess mechanisms to adapt synaptic GABA during a wide range of activity states. I hypothesized that one effective mechanism may involve regulating GABA synthesis and vesicular GABA content through modulating the supply of glutamine by System A glutamine transporters. In addition, because GABA serves critical roles during early hippocampal development, I hypothesized that the role of System A transporters at inhibitory synapses also may be developmentlally regulated. Therefore, this dissertation explored the role for System A transporters in regulating inhibitory synaptic transmission. In addition, I asked if modulation of System A surface activity dynamically regulated GABA synthesis in response to changing developmental and neuronal activity states. I now conclude that astrocytes supply interneuron terminals with glutamine for neuronal uptake by System A transporters, where supply of glutamine exists in a dynamic equilibrium with GABA synthesis and vesicle filling. I also conclude that System A transporters serve both a constitutive and activity-dependent role in modulating vesicular GABA content and

inhibitory synaptic strength. I demonstrated that synaptic depolarization up-regulates the surface activity of System A transporters and thus induces an increase in vesicular GABA content in both immature and mature hippocampus. However, because System A's basal uptake activity and therefore its constitutive contribution to vesicular GABA content diminished over the first two postnatal weeks, its role in mature hippocampus is only manifested in an activity-dependent manner. Furthermore, my results support that these constitutive and activity-dependent roles are likely mediated by the SNAT1 subtype of System A transporters. Therefore, my findings strongly support that the surface activity of SNAT1, regulated both by depolarization and by developmental cues, is the key component in a novel mechanism to dynamically link metabolic demand for GABA with vesicular GABA content and inhibitory synaptic strength. This body of research provides a framework for future studies to explore the implications of SNAT1's constitutive and activity-dependent roles in hippocampal synapse maturation, as well as in disorders characterized by chronic hyperexcitability such as epilepsy.

In this final chapter, I will provide a rationale for these conclusions, as well as discuss their physiological implications for the field. Interwoven in each section are unresolved questions that stemmed from this thesis research as well as potential future experiments to address these questions.

SNAT1 defines System A transport at interneuron terminals

Unique functional roles for SNAT1 and SNAT2

Multiples lines of evidence now support that SNAT1 is likely the main System A transporter that participates in the glu-gln-GABA pathway at interneuron terminals. First, we observed immunoreactivity for SNAT1 in only non-pyramidal neurons in area CA1 of hippocampus (Figure 17), suggesting a unique role for SNAT1 on interneurons. This expression pattern is not developmentally regulated, as it occurred in both immature (P8) and mature (P25) hippocampus. Second, preliminary data from our lab demonstrates that SNAT1 immunoreactivity positively co-localizes with GABA in P21 hippocampus (data not shown). Future studies should confirm this preliminary result and repeat this experiment in hippocampal sections from sequential postnatal ages. Lastly, in Chapter III, I demonstrated that the level of SNAT1 expression in hippocampal synaptosomes correlated with the measured uptake of [^{3}H]MeAIB (Figure 18) and with the physiological data that implicated a loss in System A's constitutive contribution to synaptic GABA (Figure 16). This correlation was not observed for SNAT2, as protein levels in immature whole hippocampal lysates were faint and increased to a low level in mature lysates.

It is now apparent that glutamatergic and GABAergic neurons likely evolved unique yet similar mechanisms to regulate synthesis of their neurotransmitters through selective expression of System A transporters. Indeed, recent immunohistochemical studies identified that glutamatergic neurons across multiple brain structures selectively express SNAT2 (Gonzalez-Gonzalez et al. 2005, Jenstad et al. 2009). Therefore,

previous studies in Chapter I that identified a MeAIB-sensitive role for glutamine in excitatory neurotransmission were likely studying glutamine transport by SNAT2. Future studies should investigate if glutamatergic neurons only require glutamine under pathological conditions (see Chapter I) and if glutamatergic neurons possible regulate SNAT2 surface activity to mediate this requirement.

Several interneuron subtypes express SNAT1

An outstanding question in the field remains that if interneurons selectively express SNAT1, is SNAT1 ubiquitously or selectively expressed amongst interneuron subtypes? Electrophysiological and preliminary immunohistochemical data indicates that in hippocampal area CA1, several interneuron subtypes that make axo-somatic and axo-dendritic synapses express SNAT1. In an effort to categorize the interneuron subtypes that express SNAT1, our lab completed co-localization experiments between SNAT1 and several neurochemical markers of interneurons, including parvalbumin (PV), somatostatin (SOM), and calbindin (CB) in P8 and P25 hippocampus (n=2 tissue sections/marker/age). At the resolution of neuronal cell bodies, a fraction of SNAT1+ cell bodies were also PV+ in P8 hippocampus (data not shown). Therefore, one sub-population appears to be PV+ basket cells, where their cell bodies localize near the pyramidale cell layer and their axons exclusively innervate the perisomatic region of CA1 pyramidal neurons. However, not all SNAT1+ neurons are PV+, indicating that more than one interneuron subtype expresses SNAT1. Data presented in Chapters II and III support that SNAT1 functions at interneuron terminals. Consequently, future

experiments must utilize techniques capable of resolving SNAT1 immunoreactivity at synaptic terminals, such as confocal microscopy.

Lastly, our electrophysiological data supports that several interneuron subtypes likely express SNAT1. Using the data presented in Figure 16, we assumed that subpopulations of synapses have similar quantal sizes and thus we grouped mIPSC amplitudes into quartiles. For example, axo-dendritic synapses may elicit smaller mIPSC amplitudes (quartiles 1 and 2) while axo-somatic synapses may elicit larger mIPSC amplitudes (quartiles 3 and 4) (Figure 20). I found that MeAIB uniformly reduced mIPSC amplitudes in each quartile in immature slices (P7-8; $p<0.05$ in each quartile), supporting that multiple interneuron subtypes express SNAT1. MeAIB does not reduce mIPSC amplitudes in any quartile in mature slices (P22-25; $p>0.05$ in each quartile). However, a noticeable pattern in Figure 19 resembles a shift towards MeAIB reducing the largest mIPSC amplitudes as development progresses. Although current data supports that a uniform loss in System A's constitutive contribution to synaptic GABA likely occurs at several subtypes of mature interneuron synapses, it will be important for future anatomical studies to confirm this conclusion.

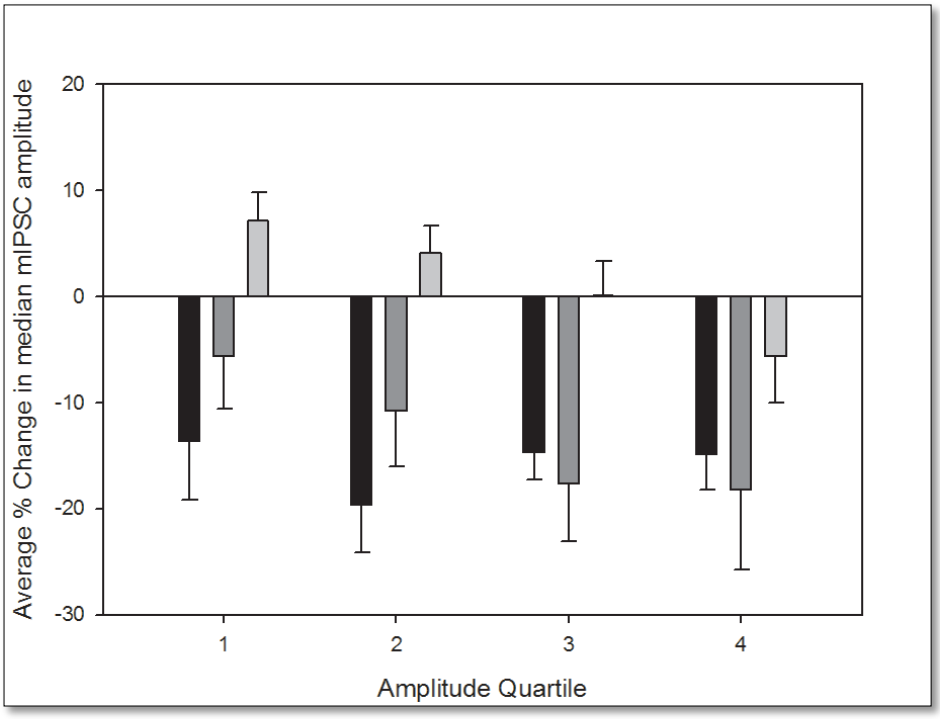

Figure 20. Effect of MeAIB on mIPSC amplitudes binned into quartiles throughout postnatal development

Comparison of the effect of 10mM MeAIB on mIPSC amplitudes reported as a % change from baseline and binned into quartiles from P7-P8 (black), P10-P12 (dark gray), or P22-25 (light gray) hippocampal slices. Error bars, SEM.

SNAT1 transports astrocytic glutamine for vesicular GABA synthesis

Data in both Chapters II and III support the conclusion that when active, SNAT1 functions within an independent substrate pathway that supplies glutamine to inhibitory terminals to maintain or to increase vesicular GABA synthesis. This pathway involves the metabolic and transport processes illustrated in Figure 21, and possibly represents only a subset of processes that enable glutamine to contribute to GABA synthesis as discussed below.

Role of EAAT2 and System N transporters

Studies in Chapter II demonstrated that EAAT2-mediated glutamate uptake by astrocytes provides one source of substrate for glutamine synthetase to synthesize glutamine (Figure 12). In addition, compensatory synthesis of astrocytic glutamate by glucose or GABA catabolism did not prevent DHK (a selective EAAT2 inhibitor) from eliminating the effect of MeAIB during resting conditions (see Figures 12 and 21). However, this does not eliminate the possibility that these pathways may contribute to astrocytic glutamate synthesis under conditions of increased synaptic activity. Therefore, future experiments could explore if inhibiting glucose or GABA catabolism eliminates the effect of MeAIB on mIPSC amplitudes following periods of increased neuronal activity.

Although EAAT2 clears synaptically released glutamate, astrocytic processes that express System N transporters ensheath inhibitory terminals both proximal and distal to glutamatergic terminals in area CA1 (Boulland et al. 2002, Cubelos et al. 2005). This

anatomical arrangement provides inhibitory terminals with a ubiquitous supply of glutamine and thus facilitates SNAT1 to regulate GABA synthesis across several interneuron subtypes. Moreover, this ubiquitous glutamine supply exists at concentrations capable of saturating SNAT1 and does not limit the constitutive contribution of glutamine to synaptic GABA (Figure 19).

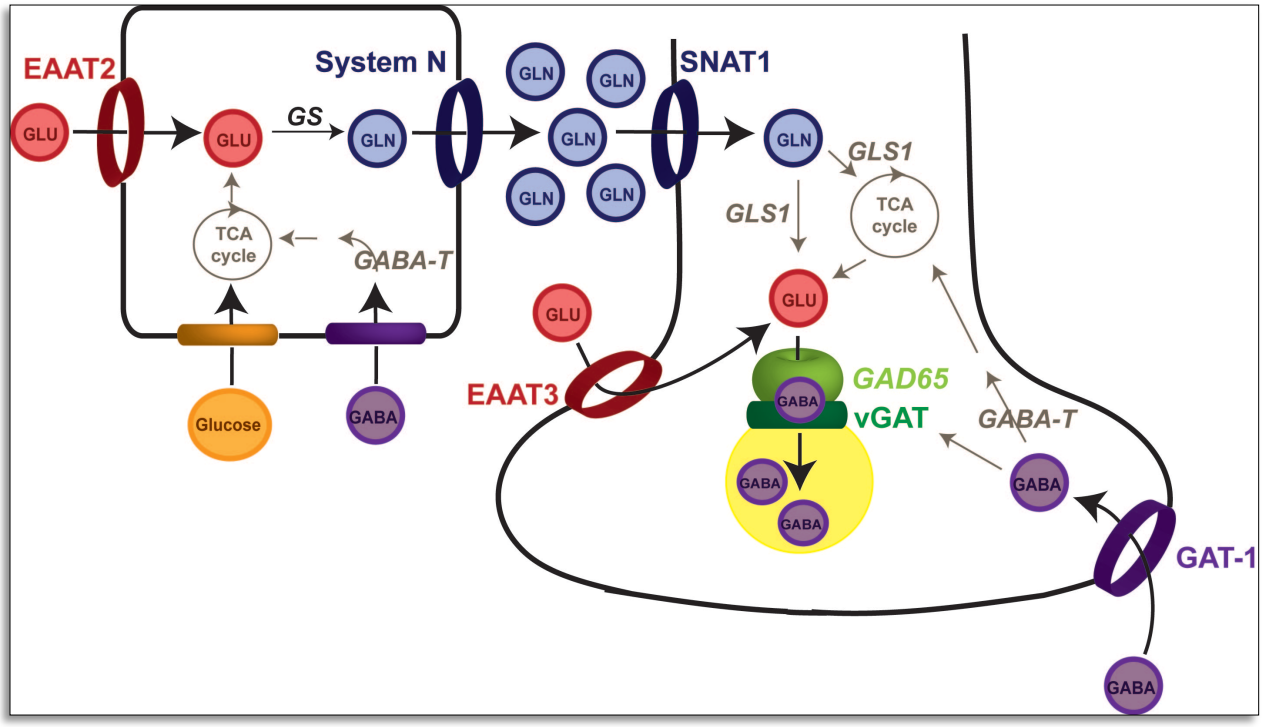

Figure 21. SNAT1 supplies interneurons with astrocytic glutamine for GABA synthesis
The schematic cartoon from Figure 6 is revised to reflect the identified role of SNAT1 in maintaining ongoing GABA synthesis. Astrocytes uptake synaptically released glutamate by EAAT2, providing substrate for glutamine synthesis by GS. System N transporters efflux glutamine for neuronal uptake by SNAT1. Neurons synthesize glutamate from glutamine through currently unconfirmed enzymatic steps (gray), providing substrate for GAD to synthesize GABA for vesicle filling. Note the studies in Chapters II and III did not address the contribution of other sources of astrocytic or neuronal glutamate to GABA synthesis (gray).

Role of GLS1

As noted previously, the role of GLS1 as the primary neuronal enzyme synthesizing glutamate from glutamine at inhibitory terminals remains controversial. Upon identifying an age-dependent reduction in the constitutive contribution of SNAT1-mediated glutamine uptake to synaptic GABA, I asked two questions regarding the role of GLS1: 1) Is GLS1 the enzyme that synthesizes neuronal glutamate from glutamine supplied by SNAT1? and 2) Is GLS1's potential role in vesicular GABA synthesis developmentally regulated? To address these questions, I examined the effect of 6-diazo-5-oxo-L-norleucine or DON, a glutaminase inhibitor, on mIPSC amplitudes in immature and mature hippocampal slices (using identical recording conditions to that reported in Chapter III). Unexpectedly, DON [6mM, 30m-1h incubation; (Sepkuty et al. 2002, Holten & Gundersen 2008)] did not reduce mIPSC amplitudes from immature slices (40 ± 10pA, n=23, v. 37 ± 9pA after DON, n=10; p=0.4, un-paired t-test). However, in mature slices, DON did slightly reduce mIPSC amplitudes (36 ± 5pA, n=14, v. 31 ± 5pA after DON, n=9; $p<0.05$, un-paired t-test) with no effect on mIPSC decay or frequency.

DON is a non-selective glutaminase inhibitor, and thus future experiments should first validate these results with a selective pharmacological inhibitor. If these results are reproducible, two possible explanations exist: First, at immature hippocampal inhibitory terminals, an enzyme besides GLS1 converts glutamine supplied by SNAT1 to glutamate. This may exist as an orphan glutaminase isozyme insensitive to DON (see Chapter I) or as an entirely different enzyme capable of deamidation through either single or multiple catalytic steps. Future electrophysiological experiments could explore the contribution of candidate enzymes through applying selective pharmacological inhibitors. Second, at

mature terminals, a source besides SNAT1 possibly supplies glutamine to serve as a substrate for GLS1. Amino acid Systems L, ASC, N, and A transport the majority of glutamine in the brain. Hippocampal astrocytes selectively express System N transporters, leaving members of Systems L and ASC, or an unidentified glutamine transporter, as candidates. A recent immunohistochemical study in hippocampus of adult mice co-localized ASCT2 with MAP2 but not NeuN, supporting a dendritic but not somatic neuronal localization (Gliddon *et al.* 2009). Although expression by interneuron terminals remains unknown, glutamine is a substrate for amino acid exchange by ASCT2 (Bak et al. 2006). ASCT2-sensitive transport is defined as glutamine transport insensitive to MeAIB and BCH (a selective substrate for System L). Future studies could explore the role of ASCT2 in GABA synthesis through two experiments: 1) Determine if neuronal terminals possess functional ASCT2 transport activity by measuring [^{3}H]glutamine uptake that is insensitive to MeAIB and BCH in hippocampal synaptosomes and 2) Examine the contribution of glutamine supplied by ASCT2 to vesicular GABA synthesis by recording the effect of MeAIB + BCH on mIPSC amplitudes.

Supply of GABAergic precursors dynamically regulates vesicular GABA content

Multiple factors determine the concentration of neurotransmitter in a synaptic vesicle, including the cytosolic concentration of neurotransmitter, the activity of the vesicular transporter, and the proton electrochemical driving force. Studies presented in Chapters II and III support the conclusion that at hippocampal interneuron terminals, the cytosolic concentration of GABA is an important determinant of vesicular GABA content. Moreover, I conclude that a dynamic equilibrium exists between the supply of GABAergic precursors and the cytosolic concentrations of newly synthesized GABA. Blocking glutamine transported by active SNAT1 robustly and rapidly decreased vesicular GABA synthesis within 5 minutes of MeAIB application under a variety of neuronal conditions (Figures 6-9, 14-15). This implies that glutamine supplied by SNAT1 must be in equilibrium with glutamate and GABA synthesis downstream. Because GAD65 and vGAT exist as part of a protein complex at synaptic vesicles, this suggests that the equilibrium between supply of precursors with the cytosolic GABA concentration likely extends to vesicle filling.

The existence of an equilibrium between glutamine supply, GABA synthesis, and vesicle filling yields several conclusions and avenues for future experiments: First, interneurons must be equipped with mechanisms to refill synaptic vesicles with GABA in order to avoid depletion of vesicular stores during their high rates of neuronal firing. Up-regulation of SNAT1's surface activity increases glutamine supply and thus increases cytosolic GABA concentrations, thereby providing one possible mechanism to sustain vesicle filling during periods of increased metabolic demands.

Second, because electrophysiological experiments examining changes in vesicular GABA content took place in the absence of synaptic activity, results from these studies suggest that vesicles do not have to be released and recycled before an effect of modulating GABA synthesis on vesicle filling is observed. This suggests that when active, glutamine supplied by SNAT1 and glutamate supplied by EAAT3 (Mathews & Diamond 2003, Stafford et al. 2009) modulate the concentration of vesicular GABA in synaptic vesicles ready to be released. These vesicles comprise the "readily releasable pool" (RRP) and their release can be evoked through application of a hyperosmotic solution. In Chapter II, I demonstrated that MeAIB reduced hyperosmotically-evoked mIPSC amplitudes by a similar magnitude as TTX-sensitive mIPSCs. In addition, previous studies demonstrate that synaptic vesicles released spontaneously and during neuronal activity belong to the same vesicle pool (Groemer & Klingauf 2007) and utilize similar modes of endocytotic uptake (Ryan *et al.* 1997). Therefore, these findings support that the dependence on glutamine supply for vesicular GABA synthesis applies to a physiologically relevant pool of vesicles released following an action potential.

Third, in Chapter III, I demonstrated that at immature interneuron terminals, glutamine supplied by SNAT1 can bi-directionally regulate vesicular GABA content (Figures 14, 15). This result implies that terminals can release both "under- and over-filled" synaptic vesicles. The cellular mechanisms that influence the extent of vesicle filling with synaptic function at central synapses remain unresolved. However, these studies support that under the neuronal activity states examined, GABAergic vesicles are not filled to static "set-points" of neurotransmitter. Instead, vesicular GABA content

fluctuates across a range of concentrations and thus is a key determinant of synaptic $[GABA]_{cleft}$ and inhibitory synaptic strength.

Activity-dependent regulation of SNAT1 surface activity

Mechanisms of synaptic plasticity modulate synaptic strength and facilitate information processing. Presynaptic control of quantal size has gained recent attention as a possible mechanism of synaptic plasticity (Edwards 2007). In Chapter III, I demonstrated that a moderate level of hyperexcitability upregulated SNAT1 surface activity to enhance vesicular GABA content and presynaptic quantal size. Whether other patterns of neuronal activity upregulate SNAT1 surface activity have not been explored. However, augmenting vesicular GABA content will only increase inhibitory synaptic strength if postsynaptic receptors remain unsaturated by synaptic GABA. For example, high frequency stimulation protocols that induce LTP presynaptically are commonly associated with a persistent activity-dependent increase in vesicle release probability. This may result in terminals releasing more than one synaptic vesicle and thus under these conditions, synaptic GABA may saturate postsynaptic $GABA_A$ receptors. Similarly, distinct patterns of neuronal activity may modulate the number of $GABA_A$ receptors expressed at the cell surface to alter their degree of saturation by synaptic GABA.

Therefore, I hypothesize that the functional implications for the activity-dependent role of SNAT1 will depend upon the degree of $GABA_A$ receptor saturation and are thus two-fold: 1) During periods of interneuron firing that result in the saturation of $GABA_A$ receptors by synaptic GABA, interneurons may continue to upregulate SNAT1 surface activity as a mechanism to maintain vesicular GABA content and thus the neurotransmitter pool of GABA. 2) During periods of interneuron firing that maintain

unsaturating conditions for $GABA_A$ receptors, upregulation of SNAT1 surface activity provides interneurons with a gain control mechanism to fine-tune GABA synthesis, vesicular GABA content, and inhibitory synaptic strength. Given the extensive axonal arbors of interneurons, small adjustments in their presynaptic input could still have a large impact on the excitability and neuronal output of principal neurons. Therefore, under relevant neuronal activity states, modulation of SNAT1 surface activity indeed defines one presynaptic mechanism that influences quantal size and thus joins existing mechanisms of synaptic plasticity that dynamically regulate synaptic strength.

From studies presented in Chapter III, I hypothesize that one cellular mechanism that models the activity-dependent increase in SNAT1-mediated glutamine uptake at both immature and mature inhibitory terminals includes the following processes (Figure 22): 1) Immature interneuron terminals express an increased amount of active SNAT1 protein relative to mature interneuron terminals, allowing SNAT1's constitutive surface activity to maintain ongoing GABA synthesis and inhibitory synaptic transmission (Figures 8,16). Synaptic depolarization may activate cellular signals to enhance glutamine's K_M for existing SNAT1 transporters at the cell surface and thus allow for an immediate increase in SNAT1 uptake upon restoring the electrochemical gradient for glutamine uptake (Figure 15). The upregulation of SNAT1's uptake activity increases glutamine supply, vesicular GABA content, and inhibitory synaptic transmission. 2) By the end of the second postnatal week, the amount of SNAT1 protein expressed in hippocampus reduces to adult levels. This leads to the question, "What is the cellular localization of SNAT1 protein in mature hippocampus and why is SNAT1 constitutively inactive?" One

hypothesized mechanism involves activation of a cellular signal towards the end of the second postnatal week that redistributes SNAT1 protein to an endsome-containing intracellular compartment. If true, synaptic depolarization likely activates cellular signals to redistribute SNAT1 at the plasma membrane surface, thereby upregulating SNAT1 uptake activity (increase in glutamine's V_{MAX} for SNAT1) to increase glutamine supply, GABA synthesis and inhibitory synaptic transmission. Although this process likely occurs during synaptic depolarization, it appears that the increase in SNAT1 uptake activity is delayed upon restoring the electrochemical gradient (Figure 15).

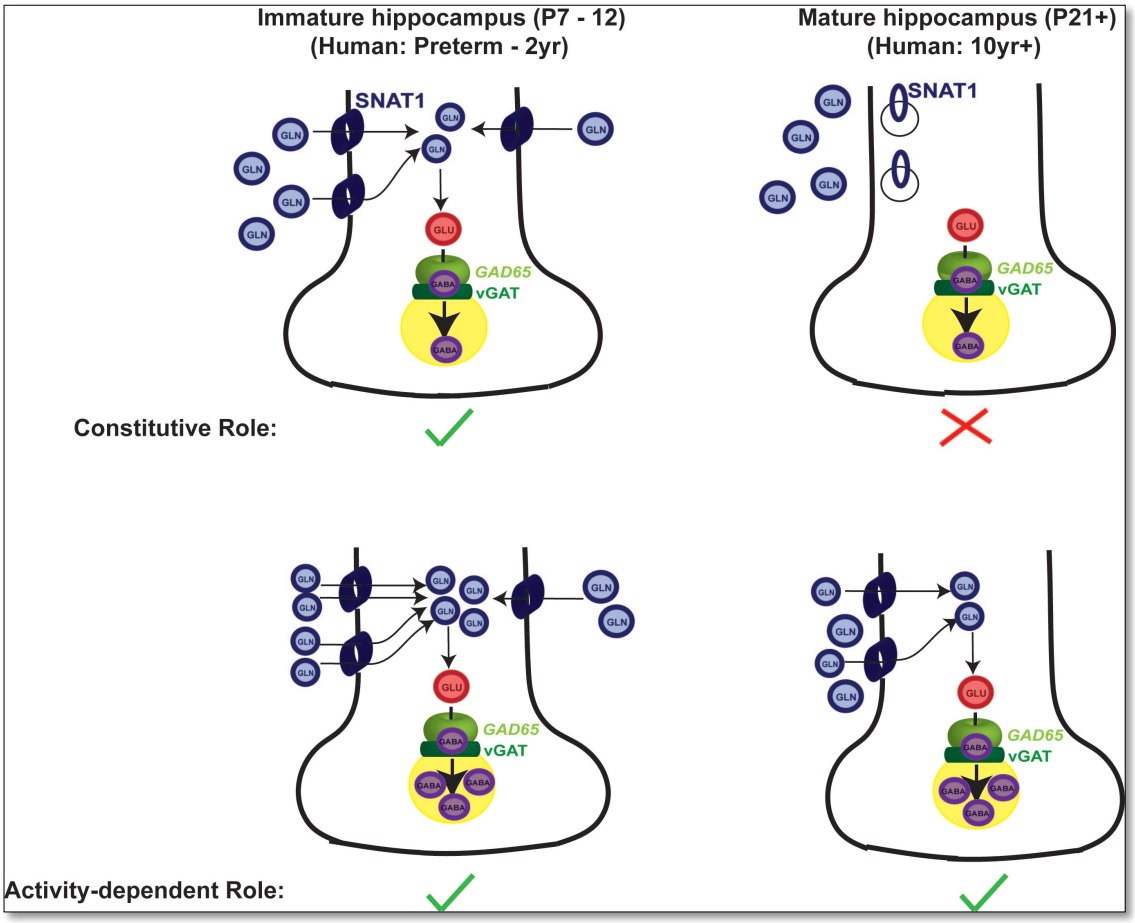

Figure 22. Age- and activity-dependent regulation of SNAT1 surface activity
A cartoon that illustrates one possible mechanism that underlies the constitutive and activity-dependent roles for SNAT1 at inhibitory terminals. *Constitutive Role,* In immature hippocampus, SNAT1 constitutively supplies inhibitory terminals with glutamine to maintain ongoing GABA synthesis. As development progresses, mature hippocampal interneurons reduce the amount of SNAT1 protein expressed. This turns off glutamine's constitutive contribution to GABA synthesis, possibly due to endocytosis of SNAT1 transporters at the cell surface. *Activity-dependent Role,* In immature hippocampus, synaptic depolarization enhances glutamine's K_M for existing SNAT1 transporters at the cell surface, thereby increasing glutamine supply and vesicular GABA content. If SNAT1 is localized within intracellular endosomes in mature interneurons, synaptic depolarization likely enhances glutamine's V_{MAX} for SNAT1 due to a trafficking-dependent mechanism. This process would increase glutamine supply and vesicular GABA content.

To test these hypothesized mechanisms, I propose addressing the questions and experiments below using the following model systems. Studies involving mature hippocampus could use slices or synaptosomes from hippocampi transduced with a recombinant adeno-associated viral vector that drives expression of SNAT1 or SNAT2 under the control of the GAD promoter (AAV-GAD-SNAT) (paired hippocampi used as control). SNAT1 antisense-transduced rats offer an additional tool to confirm its role in current physiological results, as well as to confirm its role in experiments proposed below. SNAT2 antisense-transduced rats offer a tool to selectively probe SNAT1 function with MeAIB for both future electrophysiology and uptake experiments. Unfortunately, transducing hippocampi with AAV constructs is not feasible in immature rats, as experiments cannot be complete until several weeks post-transduction. Therefore, one possibility includes applying an RNA interference approach to knockdown SNAT1 or SNAT2 expression using *in utero* electroporation (Wang & Kriegstein 2008). An alternative approach involves designing selective inhibitors for both SNAT1 and SNAT2, a critical tool for the field independent of the studies proposed below.

1) Does the high K^+-induced depolarization alter glutamine's K_M or V_{MAX} for SNAT1?

Because MeAIB is a substrate for both SNAT1 and SNAT2, I propose future studies complete the saturation kinetic analysis for SNAT1 transport ± 10mM KCl in hippocampal synaptosomes from rats that express a reduced amount of SNAT2 protein, allowing [^{3}H]MeAIB uptake to mostly reflect SNAT1 uptake activity. According to the model in Figure 22, I hypothesize that the high K^+-induced depolarization will reduce the

K_M of glutamine for SNAT1 in immature synaptosomes and increase its V_{MAX} in mature synaptosomes.

2) Does the high K^+-induced depolarization increase the surface expression of SNAT1?

Prior to completion of the uptake assays, I attempted to measure the amount of SNAT1 expressed at the cell surface ± 10mM KCl using a surface biotinylation approach in hippocampal slices. I could not detect a measurable SNAT1 protein band in the surface fraction even after exposure to 10mM KCl. However, I have recently identified a few steps in my current protocol that can be optimized, as well as redesigned the steps involving slice incubation in 10mM KCl based upon results from the uptake assays. Therefore, future experiments should re-visit this approach. According to the model in Figure 22, I hypothesize that the high K^+-induced depolarization will increase the amount of SNAT1 protein expressed in the surface fraction of mature slices. Moreover, comparing the distribution of SNAT1 protein between the intracellular and surface fractions from immature v. mature sham-treated slices will also test the hypothesis that SNAT1 protein localizes to an intracellular compartment in the absence of neuronal activity in mature hippocampus.

3) Does the high K^+-induced depolarization augment SNAT1-mediated currents?

Transport of glutamine by SNAT1 requires co-transport of sodium, thereby producing an inward current upon uptake of substrate. Because MeAIB is a selective substrate for System A transporters, MeAIB-evoked currents can be recorded from hippocampal interneurons. I hypothesize that enhancing either the K_M or V_{MAX} for

glutamine transport by SNAT1 following high K^+-induced depolarization will increase MeAIB-evoked currents. In addition, I hypothesize that in the absence of 10mM KCl, MeAIB should evoke an inward current in immature but not mature slices, providing further support for the age-dependent regulation of SNAT1 surface activity. Although this experiment may not be able to distinguish between changes in K_M or V_{MAX} ± 10mM KCl, one advantage to applying this technique is that biocytin can be injected through the recording electrode, allowing post-morphological analysis of the interneuron subtype. Further processing of the slice may enable staining for neurochemical markers and thus complement future immunohistochemical experiments to elucidate the interneuron subtypes that express functional SNAT1.

4) What are the signaling molecules that regulate SNAT1 surface activity?

To elucidate the signaling molecules that regulate SNAT1 surface activity in response to high K^+-induced depolarization, I asked if the increase in vesicular GABA content and mIPSC amplitude from electrophysiological studies was mediated by an increase in synaptic activity (i.e. glutamate) or by an increase in synaptic depolarization (i.e. calcium). If synaptic activity activated a signaling cascade that up-regulates SNAT1 surface activity, I hypothesized that TTX (0.5μM, added to 10mM KCl ACSF) would eliminate the post-stimulation increase in mIPSC amplitudes. TTX eliminated the increase in mIPSC amplitudes at 10 minutes post-stimulation in 3/6 cells (124 ± 14% of control, $p=0.1$, $n=6$) from immature slices and in 5/7 cells from mature slices (102 ± 3% of control, $p>0.05$, $n=7$). Results from this experiment imply that the cellular signals regulating SNAT1 surface activity may be developmentally regulated and involve

activation by synaptic depolarization in immature slices and by synaptic activity in mature slices. However, the increase in SNAT1-mediated uptake by 10mM KCl demonstrated that mechanisms intrinsic to synaptosomes are capable of reproducing the depolarization-induced changes observed in hippocampal slices.

Therefore, to build on these initial experiments, I would first confirm the above result by repeating the experiment, especially since the average response in immature slices appears skewed by three cells. Second, if synaptic activity is implicated (at either age), I would first explore the role of ionotropic and metabotropic glutamate receptors by repeating electrophysiological experiments completed in Chapter III using selective pharmacological antagonists ± 10mM KCl. If synaptic depolarization is implicated, I would first explore a role for extracellular calcium by repeating the electrophysiological or [^{3}H]MeAIB uptake experiments in ACSF that contains either a low concentration of calcium, a calcium chelator, or an inhibitor to voltage-gated calcium channels. Results from these proposed experiments can be confirmed by measuring MeAIB-evoked currents in immature and mature slices.

As noted in Chapter I, SNAT1 possesses three PKC consensus sequences. Both synaptic activity and synaptic depolarization could increase intracellular calcium levels and thus activate PKC. Individual PKC subtypes positively regulate the surface activity of EAAT3 in C6 glioma cells, with PKCε enhancing the K_M of glutamate for EAAT3 while PKCα enhances its V_{MAX} through a trafficking-dependent mechanism (Gonzalez et al. 2002). Therefore, a similar phenomenon may occur with SNAT1, allowing individual PKC subtypes to enhance glutamine's K_M and V_{MAX} for SNAT1. Pharmacological activators and inhibitors for various PKC subtypes exist, and thus can be applied in

electrophysiological, surface biotinylation, and synaptosomal uptake experiments ±10mM KCl to investigate if PKC is a regulator of SNAT1 surface activity.

Age-dependent regulation of SNAT1 surface activity

Studies in Chapter III support the conclusion that towards the end of the second postnatal week, inhibitory terminals reduce the amount of SNAT1 protein expressed and the basal uptake activity of SNAT1 to adult levels, which both eliminate glutamine supplied by SNAT1 from constitutively contributing to vesicular GABA synthesis. What is the functional role of this age-dependent regulation of SNAT1 surface activity? As noted in Chapter I (Figure 2), functional maturation of the first excitatory synapses requires GABA-induced depolarization and thus occurs during P0-P12, prior to reversal of the chloride concentration gradient and loss of GABA's "depolarizing" activity. In order for GABA to serve in this capacity, interneurons must possess metabolic or synthetic pathways to maintain synaptic GABA levels during this developmental window. Inhibitory synapses likely encounter little extracellular glutamate supply during synapse maturation, yet extracellular glutamine exists at concentrations capable of saturating SNAT1. Therefore, I hypothesize that SNAT1's constitutive surface activity provides interneurons with a mechanism to maintain ongoing GABA synthesis and synaptic GABA levels during this developmental window. This hypothesis is supported by the correlation between the postnatal age that chloride's concentration gradient reverses with the postnatal age that SNAT1's constitutive surface activity is lost (both by P14). By P14, a sufficient density of mature excitatory synapses have formed and thus GABA itself functions within a feedback loop to initiate reversal of chloride's concentration gradient and switch its role from an "excitatory" to an inhibitory neurotransmitter. Consequently, this feedback loop may also give rise to a retrograde

cellular signal that turns off SNAT1's constitutive surface activity in an effort to transition GABA synthesis from constitutive during P0-P12 to activity-dependent as GABA assumes its role as an inhibitory neurotransmitter.

Elucidating the mechanism that underlies the activity-dependent regulation of SNAT1 surface activity will provide insight to the age-dependent regulatory mechanism. If synaptic depolarization redistributes SNAT1 to the plasma membrane in mature slices, then a cellular signal likely turns off SNAT1's constitutive surface activity through stimulating SNAT1 endocytosis towards the end of the second postnatal week. In Figure 19, I demonstrated that developing inhibitory synapses do not down-regulate constitutive activity of the entire glu-gln-GABA pathway, indicating that the cellular signal selectively targets SNAT1. To further elucidate the age-dependent regulatory mechanism, I propose addressing the questions and experiments below.

1) What is the cellular distribution pattern of SNAT1 protein during postnatal development?

Multiple methodological approaches could be applied to address this question. As noted in the previous section, one approach involves measuring the amount of SNAT1 protein expressed in the surface and intracellular fractions in immature and mature hippocampal slices using surface biotinylation assays. In addition, hippocampal synaptosomes could be further fractioned into plasma and vesicle membrane fractions and probed for SNAT1 protein. If SNAT1's cellular distribution pattern switches from a surface to predominantly an intracellular localization, I hypothesize that SNAT1 will localize to the surface and plasma membrane fractions in immature slices and synaptosomes and to the

intracellular and vesicular membrane fractions in mature slices and synaptosomes, respectfully. These experiments could be completed at multiple postnatal timepoints between P12 and P21 in order to narrow the developmental window in which SNAT1 protein redistribution may occur. Upon narrowing the developmental window, the subcellular distribution pattern of SNAT1 immunoreactivity could be confirmed through electron microscopy. Conversely, if the age-dependent mechanism involves mature terminals that express inactive SNAT1 transporters at the cell surface, results from the above experiments would provide further support for this mechanism as well.

2) If the mechanism involves SNAT1 endocytosis, does blocking this process retain functional SNAT1 protein at the cell surface?

To address this question, experiments must first identify the mechanism of endocytosis and then determine if inhibition of key proteins involved retain SNAT1's constitutive surface activity. This can be addressed through electrophysiological (± MeAIB during resting conditions) and surface activity (basal [^{3}H]MeAIB uptake, surface levels, MeAIB-evoked currents) measurements in slices or synaptosomes acutely exposed to inhibitors of these endocytosis mechanisms. An alternative approach involves applying the same RNAi strategy using *in utero* electroporation to knockdown expression of these targeted proteins and repeating the above experiments.

3) What are the signaling molecules that turn off SNAT1's constitutive surface activity?

In addition to GABA, PKC may also function as a cellular signal to turn off SNAT1's constitutive surface activity. Although PKC increases the surface activity of EAAT3, it

also reduces the surface activity of most transporters in the SLC6 gene family, including GAT-1 (Melikian 2004). Therefore, experiments proposed above (#2) could be repeated with pharmacological inhibitors to PKC. Likewise, if the mechanism requires GABA-induced depolarization to liberate a retrograde signal, experiments that measure SNAT1 surface activity could be repeated in the presence of $GABA_A$ receptor antagonists.

4) Does SNAT1 play a role in synapse maturation during early postnatal development? This question can be addressed by examining three primary parameters in wildtype hippocampi and hippocampi from P0-P12 rats that express reduced SNAT1 protein (RNAi approach using *in utero* electroporation): GDPs, $GABA_A$ and AMPA receptor-mediated sPSCs, and the neuronal morphology of CA1 pyramidal neurons. As noted in Chapter I, GDPs elicited by activation of $GABA_A$ receptors provide the primary source of neuronal activity during early postnatal development (P0-P8). If SNAT1 activity contributes to the neurotransmitter pool of GABA that elicits these GDPs, I hypothesize that bath application of MeAIB will reduce the amplitude, duration, or frequency of these GDPs and spontaneous $GABA_A$ receptor-mediated PSCs in wildtype slices. Similar effects should be observed in slices that express a reduced amount of SNAT1 protein. Likewise, I hypothesize that reducing SNAT1 protein expression will either eliminate or reduce the frequency of AMPA receptor-mediated sPSCs recorded from CA1 pyramidal neurons as a consequence of disrupting glutamatergic synapse maturation. In addition, it may disrupt the neuronal morphology of CA1 pyramidal neurons, as GABA-induced depolarization contributes to the maturation of cortical pyramidal neuron dendrites. An alternative approach involves repeating the above experiments following daily injections

of a SNAT1-selective inhibitor to either the pregnant mothers during late embryonic development or to their newly born rat pups.

Because neurons express functional GABA and glutamate receptors at birth, GABA serves several trophic roles prior to the formation of synapses, including in the proliferation of cortical progenitor cells and in neuronal growth and migration (Wang & Kriegstein 2009). Addition of the $GABA_A$ receptor agonist, Muscimol (100μM), increases neuronal growth in immature (P0-P7) but not mature (P21+) hippocampal neuronal cultures (Marty *et al.* 1996) due to activation of downstream cellular signals that induces transcription of BDNF gene expression (Berninger *et al.* 1995). BDNF increases SNAT1 uptake activity in cultured neocortical neurons (Burkhalter et al. 2007) and is a key player in LTP_{GABA} at immature hippocampal inhibitory synapses (Gubellini *et al.* 2005) (see Chapter I). It would be interesting to determine if BDNF modulates SNAT1 surface activity to facilitate BDNF and GABA's role in neuronal growth during early postnatal development. SNAT1 expression in rat hippocampal CA1 area is detected as early as E17 (Weiss *et al.* 2003), but future immunohistochemical studies should examine its neuronal localization at both embryonic and postnatal ages.

In addition, in order for GABA to serve these trophic roles, interneurons must possess mechanisms to release GABA prior to the formation of synapses. In P0 hippocampal slices, $GABA_A$ receptors elicit both spontaneous and evoked "early slow currents" that are partially sensitive to inhibitors of voltage-gated channels calcium yet insensitive to inhibitors of SNARE-dependent vesicular exocytosis mechanisms (Demarque *et al.* 2002). Therefore, interneurons likely employ nonvesicular or SNARE-

independent vesicular exocytosis mechanisms to release GABA during early postnatal development. It would be interesting to determine if bath application of MeAIB reduces these $GABA_A$ receptor-mediated "early slow currents" in P0 hippocampal slices. If true, it would support that SNAT1 supplies interneurons with glutamine to synthesize GABA for nonvesicular release and possibly functions to facilitate GABA's multiple trophic roles during early postnatal development.

Homeostatic regulation of synaptic GABA

A central conclusion to this thesis research is that modulation of glutamine supply by SNAT1's surface activity dynamically regulates vesicular GABA synthesis and thus contributes to the homeostatic regulation of synaptic GABA. This conclusion further supports the existing view that interneurons do not solely rely on GABA reuptake to maintain vesicular GABA content. In Chapter III, I demonstrated that inhibition of GAD with 3-MPA reduced mIPSC amplitudes from immature and mature slices (Figure 18), indicating that interneurons do not down-regulate the GABA synthetic protein machinery along with the constitutive surface activity of SNAT1. Several intriguing questions emerged from this experiment, including: 1) Since SNAT1 is constitutively active in immature slices and the extent of mIPSC amplitude reduction by 3-MPA appears greater than the effect of MeAIB alone, does both SNAT1 and EAAT3 activity contribute to ongoing vesicular GABA synthesis?; 2) Similarly, since SNAT1 is not constitutively active during resting conditions in mature hippocampus, is 3-MPA inhibiting GABA synthesis from glutamate supplied by EAAT3?; 3) Lastly, why does inhibiting GAD activity not completely block synaptic inhibition?

To address the first two questions, I examined the combined effect of MeAIB (10mM) + THA (300μM), a non-selective EAAT substrate that inhibits glutamate transport. If the effect of 3-MPA reflects differing contributions of glutamine and glutamate supply to vesicular GABA synthesis, I hypothesized that MeAIB + THA should reduce mIPSC amplitudes by a degree comparable to 3-MPA at the respective postnatal age. Unexpectedly, a 5m bath application of MeAIB + THA did not reduce

mIPSC amplitudes in either immature (93 ± 7% of control, n=8) or mature (99 ± 9% of control, n=3) slices. A similar result was obtained after a 50m bath application in immature slices (data not shown). One possible explanation for these findings is that a compensatory increase in vesicular GABA content by a SNAT1-EAAT3 independent mechanism masked an actual reduction in GABA synthesis by MeAIB + THA. Either reuptake of GABA through GAT-1 or GABA synthesis through TCA cycle intermediates (glutamate synthesized from α-ketoglutarate) could define this compensatory mechanism. Future experiments could evaluate the role of GAT-1 and the TCA cycle as a potential compensatory mechanism by repeating the above experiment with the additional presence of appropriate pharmacological inhibitors. As noted in the third question above, inhibiting GAD activity does not completely abolish synaptic inhibition. Two possible explanations for this observation are: 1) Under the specified conditions, 3-MPA did not completely inhibit GAD activity. Because 3-MPA inhibits GAD by competing with glutamate for the enzyme, the 1h slice incubation in 3-MPA prior to measuring mIPSCs likely raised cytosolic glutamate levels and thus could diminish the degree of GAD inhibition by 3-MPA. 2) As noted above, a compensatory mechanism that involves vesicle filling from reuptake of pre-existing GABA by GAT-1 may prevent complete abolishment of synaptic inhibition.

These findings reveal that homeostatic regulation of synaptic GABA is a complex process and is likely governed by several synthetic and metabolic pathways that each contribute to vesicular GABA content under specified neuronal conditions. For example, in immature hippocampus, the glu-gln-GABA pathway appears to exist as the preferring mechanism to maintain vesicular GABA content during resting conditions, although

reuptake of preexisting GABA is expected to also contribute. GABA synthesized from glutamate supplied by EAAT3 may contribute to vesicular GABA content at a subpopulation of interneuron terminals (axo-dendritic synapses) and only under neuronal conditions that reduce glutamate clearance by EAAT2. Upon conditions that increase neuronal activity in immature hippocampus, both SNAT1 (Chapter III) and EAAT3 (Mathews & Diamond 2003) contribute to augmenting vesicular GABA content, while the contribution by GAT-1 remains unknown. In mature hippocampus, active GABA synthesis appears to occur during resting conditions, yet glutamine supplied by SNAT1 does not contribute to this process. For reasons stated above, glutamate supplied by EAAT3 likely is not the primary pathway. In addition to GABA synthesis, reuptake of preexisting GABA by GAT-1 does appear to contribute during resting conditions. Similar to immature hippocampus, depolarization of mature interneuron terminals allows SNAT1 to augment vesicular GABA content and future experiments should investigate a possible contribution by GAT-1 and EAAT3. It will be important for future experiments to investigate the proposed contributions above in order to provide a full understanding of the mechanisms that maintain homeostatic regulation of synaptic GABA. In addition, the hippocampus expresses varying amounts of GAT-1 and EAAT3 protein throughout postnatal development (Figure 16). Whether these developmental changes occur at interneuron terminals to impact the homeostatic regulation of synaptic GABA remains unknown and thus should be a focus of future experiments.

Role of SNAT1 and GABA in Seizures and Epilepsy

Hippocampus

Epilepsy is a complex neurological disorder characterized by recurrent seizures induced by external (brain trauma, infection, or stroke) or internal (genetic mutations) insults. In temporal lobe epilepsy (TLE), recurrent seizures arise from one or both temporal lobes and the hippocampus defines one seizure locus in mesial TLE (mTLE). Hippocampal sclerosis defines one pathology of adult mTLE, characterized by atrophy, glial proliferation, and preferential loss of neurons in areas CA1, CA3, and the dentate hilus. Impaired energy metabolism due to altered levels of glutamate, glutamine, and GABA may partially mediate these pathologies. Using *in vivo* microdialysis, the hippocampi of medication-resistant epileptic patients contain over a four-fold increase in extracellular glutamate levels and slightly reduced glutamine levels compared to nonepileptogenic patients (Cavus *et al.* 2005). Both a decrease in EAAT2 immunoreactivity (Proper *et al.* 2002) and GS activity and protein expression (Eid *et al.* 2004) in hippocampus of TLE patients may explain the high and low extracellular glutamate and glutamine levels, respectively.

The slightly reduced glutamine levels in TLE patients may also affect the synthesis and extracellular levels of GABA. A study in patients with complex partial epilepsy correlated poor seizure control with low brain GABA levels in brain regions outside the hippocampal seizure focus (Petroff *et al.* 1996). Immunocytochemical studies following *in vivo* seizure induction reveal reduced GABA-like immunoreactivity in synaptic terminals of hippocampal interneurons, suggesting neurotransmitter depletion

due to inadequate GABA synthesis (Meldrum *et al.* 1987). Consistent with this finding, both GAD65 KO mice (Kash et al. 1997) and EAAT3 antisense-treated rats (Sepkuty et al. 2002) develop epilepsy, demonstrating that increased demand for GABA must be met with increased synthesis. Two intriguing questions emerge from these studies: 1) Does modulation of SNAT1 surface activity provide neuroprotection during periods of hyperexcitability? and 2) Conversely, would loss of SNAT1 function lead to hyperexcitability and seizure-like activity?

Several studies have demonstrated that hyperexcitability and acute ictal-like activity (a characteristic of seizures) can be induced in hippocampal slices by elevating extracellular potassium (Traynelis & Dingledine 1988, Jensen & Yaari 1997, Dzhala & Staley 2003). In P10-P20 hippocampal-entorhinal cortex slices, elevating the extracellular potassium concentration to 8.5mM induced interictal epileptiform discharges (IEDs; brief bursts of synchronous population activity) measured by extracellular field recordings in area CA3 that spread bi-directionally to area CA1 and the dentate hilus region (Dzhala & Staley 2003). After 2-20 minutes of perfusing high potassium, IEDs evolved into ictal-like activity (prolonged epochs of population activity). I induced a more moderate level of hyperexcitability by elevating the extracellular potassium concentration to 10mM, with the goal of depolarizing neurons, increasing synaptic activity and creating a higher demand for synaptic GABA. High K^+-induced depolarization increased SNAT1 surface activity and thus vesicular GABA content at inhibitory terminals, demonstrating that inhibition is not only maintained, but enhanced to counterbalance increased excitatory drive. Furthermore, it provides evidence that SNAT1 may indeed play a neuroprotective role during periods of acute hyperexcitability.

To further elucidate a neuroprotective role for SNAT1, the following questions can be addressed in future experiments: **1)** During IEDs and ictal-like activity, do interneurons upregulate SNAT1 surface activity to maintain or increase vesicular GABA content as a compensatory response to maintain or augment synaptic inhibition, respectfully? Given the similarities between mine and Dzhala and Staley's experimental conditions, future experiments can focus on first replicating the induction of IEDs and their transition to ictal-like activity in hippocampal-entorhinal cortical slices by elevating extracellular potassium. After recording IEDs and ictal-like activity with an extracellular recording electrode, mIPSCs could be recorded from CA1 pyramidal neurons to assess changes in vesicular GABA content. MeAIB-evoked currents could also be recorded from area CA1 interneurons to assess changes in SNAT1 surface activity. In addition, if SNAT1 surface activity is indeed increased, it will be important to investigate the duration of this upregulation. This will determine whether interneurons acutely or chronically upregulate SNAT1 surface activity in order to meet different metabolic demands. **2)** What is the cellular mechanism that potentially upregulates SNAT1 surface activity following seizure-like activity? This question can be addressed by first investigating the role of cellular signals identified in the activity-dependent regulation of SNAT1 surface activity through similar experiments proposed above. **3)** Do hippocampal slices from rats that overexpress SNAT1 lead to a reduced frequency or prolonged onset of IEDs, ictal-like activity, or the transition between the two patterns? Conversely, do rats that express a reduced amount of SNAT1 protein develop spontaneous seizures?

If the above experiments support that modulation of SNAT1 surface activity indeed provides a neuroprotective effect during periods of hyperexcitability and seizure-like activity, it would be interesting to explore SNAT1 function in several transgenic mouse models of human epilepsy (upon controlling for differences in species). The majority of these transgenic mouse models are characterized by loss- or gain-of-function mutations in voltage- and ligand-gated ion channels that appear to recapitulate several types or syndromes of human epilepsy (Bialer & White). For example, a gain-of-function mutation in a voltage-gated sodium channel leads to hyperexcitability and generalized epilepsy febrile seizures plus (GEFS+; febrile seizures during childhood progress to generalized seizures in adults) (Meisler & Kearney 2005). Likewise, loss-of-function mutations in distinct voltage-gated sodium, calcium, or potassium channels model temporal lobe epilepsy (Bialer & White). Whether interneurons modulate either the age- or activity-dependent regulatory mechanisms that govern SNAT1 surface activity in response to chronic periods of hyperexcitability could be explored through previously proposed electrophysiological and surface activity measurements in hippocampal slices from these transgenic mice.

Thalamus

Similar to hippocampal circuitry, dysfunction of thalamocortical circuits allows the thalamus to evolve as a seizure locus for specific epilepsy subtypes. The reticular thalamic nucleus encapsulates multiple thalamic nuclei and links the thalamus to the cerebral cortex (Pinault 2004). Thalamic reticular neurons (TRNs) are exclusively GABAergic interneurons, and extend their dendrites in multiple planes and in multiple

thalamic tiers, while their axons project to several thalamic nuclei. Thalamocortical and corticalthalamic inputs define the primary glutamatergic inputs to TRNs. Cholinergic, GABAergic, and monoaminergic modulatory inputs also innervate TRNs. These morphological features enable some TRNs and TCs to form a reciprocal circuit and therefore TRNs provide both feedback and lateral inhibition to TCs (Pinault 2004). This reciprocal circuit generates both sleep-related spindles (6-14Hz) and under certain conditions, epileptic oscillations (3Hz synchronized spike-and-wave discharge) associated with absence seizures (McCormick & Bal 1997).

Studies presented in Chapters II and III focused on SNAT1 function in one sub-section of one brain region. However, recent evidence supports that the roles for SNAT1 in synaptic inhibition may be conserved amongst other brain regions, particularly in the reticular thalamic nucleus. First, thalamic astrocytes appear to exclusively express GAT-1 independently of postnatal age, indicating that TRNs heavily rely on supply of glutamate or glutamine to maintain vesicular GABA synthesis (Vitellaro-Zuccarello *et al.* 2003). Second, preliminary immunohistochemical data from our lab demonstrate that the reticular thalamic nucleus highly expresses SNAT1 protein throughout postnatal development in rat. These findings support the hypothesis that astrocytes may supply glutamine for neuronal uptake by SNAT1 on TRNs, thereby providing one mechanism to maintain vesicular GABA synthesis and inhibitory synaptic transmission in the thalamus.

A recent study explored the role of glutamine in maintaining both sleep-related spindles and epileptic oscillations (induced by antagonizing $GABA_A$ receptors) in P11-P15 horizontal thalamic slices (Bryant et al. 2009). Maintaining epileptic but not sleep-related spindle oscillations required glutamine supplied by System A transporters,

supporting an activity-dependent role for glutamine in sustaining hyperexcitability of TC neurons. Because induction of epileptiform oscillations required blockade of $GABA_A$ receptors, this study did not explore the contribution of glutamine to inhibitory neurotransmission and thus could be a focus for future experiments. I hypothesize that TRNs may modulate SNAT1 surface activity as a mechanism to maintain or increase vesicular GABA content and inhibitory synaptic transmission during these epileptiform oscillations. In addition, because TRN terminals appear to not utilize GABA reuptake by GAT-1 as a mechanism to maintain vesicular GABA content, it would also be interesting to determine if SNAT1 plays a constitutive role in GABA synthesis. If true, this would support that the primary role for SNAT1 evolved to link the activity-related demands of GABA to its rate of synthesis and thus maintain synaptic GABA homeostasis for interneurons across multiple brain regions.

REFERENCES

Apparsundaram, S., Sung, U., Price, R. D. and Blakely, R. D. (2001) Trafficking-dependent and -independent pathways of neurotransmitter transporter regulation differentially involving p38 mitogen-activated protein kinase revealed in studies of insulin modulation of norepinephrine transport in SK-N-SH cells. *The Journal of Pharmacology and Experimental Therapeutics,* 299, 666-677.

Bacci, A., Sancini, G., Verderio, C., Armano, S., Pravettoni, E., Fesce, R., Franceschetti, S. and Matteoli, M. (2002) Block of glutamate-glutamine cycle between astrocytes and neurons inhibits epileptiform activity in hippocampus. *Journal of Neurophysiology,* 88, 2302-2310.

Bak, L. K., Schousboe, A. and Waagepetersen, H. S. (2006) The glutamate/GABA-glutamine cycle: aspects of transport, neurotransmitter homeostasis and ammonia transfer. *Journal of Neurochemistry,* 98, 641-653.

Ben-Ari, Y., Gaiarsa, J. L., Tyzio, R. and Khazipov, R. (2007) GABA: a pioneer transmitter that excites immature neurons and generates primitive oscillations. *Physiological Reviews,* 87, 1215-1284.

Berger, U. V. and Hediger, M. A. (1998) Comparative analysis of glutamate transporter expression in rat brain using differential double in situ hybridization. *Anat Embryol (Berl),* 198, 13-30.

Bialer, M. and White, H. S. Key factors in the discovery and development of new antiepileptic drugs. *Nat Rev Drug Discov,* 9, 68-82.

Bohme, I., Rabe, H. and Luddens, H. (2004) Four amino acids in the alpha subunits determine the gamma-aminobutyric acid sensitivities of GABAA receptor subtypes. *The Journal of Biological Chemistry,* 279, 35193-35200.

Bormann, J. (1988) Electrophysiology of GABAA and GABAB receptor subtypes. *Trends Neurosci,* 11, 112-116.

Bormann, J. and Feigenspan, A. (1995) GABAC receptors. *Trends Neurosci,* 18, 515-519.

Boulland, J. L., Osen, K. K., Levy, L. M., Danbolt, N. C., Edwards, R. H., Storm-Mathisen, J. and Chaudhry, F. A. (2002) Cell-specific expression of the glutamine transporter SN1 suggests differences in dependence on the glutamine cycle. *The European Journal of Neuroscience,* 15, 1615-1631.

Bragina, L., Marchionni, I., Omrani, A., Cozzi, A., Pellegrini-Giampietro, D. E., Cherubini, E. and Conti, F. (2008) GAT-1 regulates both tonic and phasic GABA(A) receptor-mediated inhibition in the cerebral cortex. *Journal of Neurochemistry,* 105, 1781-1793.

Broer, A., Albers, A., Setiawan, I., Edwards, R. H., Chaudhry, F. A., Lang, F., Wagner, C. A. and Broer, S. (2002) Regulation of the glutamine transporter SN1 by extracellular pH and intracellular sodium ions. *The Journal of Physiology,* 539, 3-14.

Broer, S. and Brookes, N. (2001) Transfer of glutamine between astrocytes and neurons. *Journal of Neurochemistry,* 77, 705-719.

Bryant, A. S., Li, B., Beenhakker, M. P. and Huguenard, J. R. (2009) Maintenance of thalamic epileptiform activity depends on the astrocytic glutamate-glutamine cycle. *Journal of Neurophysiology,* 102, 2880-2888.

Burkhalter, J., Fiumelli, H., Erickson, J. D. and Martin, J. L. (2007) A critical role for system A amino acid transport in the regulation of dendritic development by brain-derived neurotrophic factor (BDNF). *The Journal of Biological Chemistry,* 282, 5152-5159.

Caillard, O., Ben-Ari, Y. and Gaiarsa, J. L. (1999) Long-term potentiation of GABAergic synaptic transmission in neonatal rat hippocampus. *The Journal of Physiology,* 518 (Pt 1), 109-119.

Cavelier, P. and Attwell, D. (2005) Tonic release of glutamate by a DIDS-sensitive mechanism in rat hippocampal slices. *The Journal of Physiology,* 564, 397-410.

Cavus, I., Kasoff, W. S., Cassaday, M. P., Jacob, R., Gueorguieva, R., Sherwin, R. S., Krystal, J. H., Spencer, D. D. and Abi-Saab, W. M. (2005) Extracellular metabolites in the cortex and hippocampus of epileptic patients. *Annals of Neurology,* 57, 226-235.

Chaudhry, F. A., Reimer, R. J. and Edwards, R. H. (2002) The glutamine commute: take the N line and transfer to the A. *The Journal of Cell Biology,* 157, 349-355.

Chaudhry, F. A., Reimer, R. J., Krizaj, D., Barber, D., Storm-Mathisen, J., Copenhagen, D. R. and Edwards, R. H. (1999) Molecular analysis of system N suggests novel physiological roles in nitrogen metabolism and synaptic transmission. *Cell,* 99, 769-780.

Cobb, S. R., Buhl, E. H., Halasy, K., Paulsen, O. and Somogyi, P. (1995) Synchronization of neuronal activity in hippocampus by individual GABAergic interneurons. *Nature,* 378, 75-78.

Conti, F. and Melone, M. (2006) The glutamine commute: lost in the tube? *Neurochemistry International,* 48, 459-464.

Couve, A., Moss, S. J. and Pangalos, M. N. (2000) GABAB receptors: a new paradigm in G protein signaling. *Molecular and Cellular Neurosciences,* 16, 296-312.

Cubelos, B., Gonzalez-Gonzalez, I. M., Gimenez, C. and Zafra, F. (2005) Amino acid transporter SNAT5 localizes to glial cells in the rat brain. *Glia,* 49, 230-244.

Danglot, L., Triller, A. and Marty, S. (2006) The development of hippocampal interneurons in rodents. *Hippocampus,* 16, 1032-1060.

Demarque, M., Represa, A., Becq, H., Khalilov, I., Ben-Ari, Y. and Aniksztejn, L. (2002) Paracrine intercellular communication by a Ca2+- and SNARE-independent release of GABA and glutamate prior to synapse formation. *Neuron,* 36, 1051-1061.

Dzhala, V. I. and Staley, K. J. (2003) Transition from interictal to ictal activity in limbic networks in vitro. *J Neurosci,* 23, 7873-7880.

Edwards, R. H. (2007) The neurotransmitter cycle and quantal size. *Neuron,* 55, 835-858.

Eid, T., Thomas, M. J., Spencer, D. D. et al. (2004) Loss of glutamine synthetase in the human epileptogenic hippocampus: possible mechanism for raised extracellular glutamate in mesial temporal lobe epilepsy. *Lancet,* 363, 28-37.

Farrant, M. and Nusser, Z. (2005) Variations on an inhibitory theme: phasic and tonic activation of GABA(A) receptors. *Nature Reviews,* 6, 215-229.

Ferguson, S. M., Savchenko, V., Apparsundaram, S., Zwick, M., Wright, J., Heilman, C. J., Yi, H., Levey, A. I. and Blakely, R. D. (2003) Vesicular localization and activity-dependent trafficking of presynaptic choline transporters. *J Neurosci,* 23, 9697-9709.

Freund, T. F. and Buzsaki, G. (1996) Interneurons of the hippocampus. *Hippocampus,* 6, 347-470.

Fricke, M. N., Jones-Davis, D. M. and Mathews, G. C. (2007) Glutamine uptake by System A transporters maintains neurotransmitter GABA synthesis and inhibitory synaptic transmission. *Journal of Neurochemistry,* 102, 1895-1904.

Gaiarsa, J. L., Tseeb, V. and Ben-Ari, Y. (1995) Postnatal development of pre- and postsynaptic GABAB-mediated inhibitions in the CA3 hippocampal region of the rat. *Journal of Neurophysiology,* 73, 246-255.

Gliddon, C. M., Shao, Z., LeMaistre, J. L. and Anderson, C. M. (2009) Cellular distribution of the neutral amino acid transporter subtype ASCT2 in mouse brain. *Journal of Neurochemistry,* 108, 372-383.

Glykys, J. and Mody, I. (2007) Activation of GABAA receptors: views from outside the synaptic cleft. *Neuron,* 56, 763-770.

Gonzalez, M. I., Kazanietz, M. G. and Robinson, M. B. (2002) Regulation of the neuronal glutamate transporter excitatory amino acid carrier-1 (EAAC1) by different protein kinase C subtypes. *Molecular Pharmacology,* 62, 901-910.

Gonzalez-Gonzalez, I. M., Cubelos, B., Gimenez, C. and Zafra, F. (2005) Immunohistochemical localization of the amino acid transporter SNAT2 in the rat brain. *Neuroscience,* 130, 61-73.

Grewal, S., Defamie, N., Zhang, X., De Gois, S., Shawki, A., Mackenzie, B., Chen, C., Varoqui, H. and Erickson, J. D. (2009) SNAT2 amino acid transporter is regulated by amino acids of the SLC6 gamma-aminobutyric acid transporter subfamily in neocortical neurons and may play no role in delivering glutamine for glutamatergic transmission. *The Journal of Biological Chemistry,* 284, 11224-11236.

Groemer, T. W. and Klingauf, J. (2007) Synaptic vesicles recycling spontaneously and during activity belong to the same vesicle pool. *Nature Neuroscience,* 10, 145-147.

Gubellini, P., Ben-Ari, Y. and Gaiarsa, J. L. (2005) Endogenous neurotrophins are required for the induction of GABAergic long-term potentiation in the neonatal rat hippocampus. *J Neurosci,* 25, 5796-5802.

Hartmann, K., Bruehl, C., Golovko, T. and Draguhn, A. (2008) Fast homeostatic plasticity of inhibition via activity-dependent vesicular filling. *PloS one,* 3, e2979.

Holten, A. T. and Gundersen, V. (2008) Glutamine as a precursor for transmitter glutamate, aspartate and GABA in the cerebellum: a role for phosphate-activated glutaminase. *Journal of Neurochemistry,* 104, 1032-1042.

Hsu, C. C., Davis, K. M., Jin, H. et al. (2000) Association of L-glutamic acid decarboxylase to the 70-kDa heat shock protein as a potential anchoring mechanism to synaptic vesicles. *The Journal of Biological Chemistry,* 275, 20822-20828.

Jacob, T. C., Moss, S. J. and Jurd, R. (2008) GABA(A) receptor trafficking and its role in the dynamic modulation of neuronal inhibition. *Nature Reviews,* 9, 331-343.

Jensen, M. S. and Yaari, Y. (1997) Role of intrinsic burst firing, potassium accumulation, and electrical coupling in the elevated potassium model of hippocampal epilepsy. *Journal of Neurophysiology,* 77, 1224-1233.

Jenstad, M., Quazi, A. Z., Zilberter, M. et al. (2009) System A transporter SAT2 mediates replenishment of dendritic glutamate pools controlling retrograde signaling by glutamate. *Cereb Cortex,* 19, 1092-1106.

Jin, H., Wu, H., Osterhaus, G. et al. (2003) Demonstration of functional coupling between gamma -aminobutyric acid (GABA) synthesis and vesicular GABA transport into synaptic vesicles. *Proceedings of the National Academy of Sciences of the United States of America,* 100, 4293-4298.

Jones, M. V., Jonas, P., Sahara, Y. and Westbrook, G. L. (2001) Microscopic kinetics and energetics distinguish GABA(A) receptor agonists from antagonists. *Biophysical Journal,* 81, 2660-2670.

Jones, M. V., Sahara, Y., Dzubay, J. A. and Westbrook, G. L. (1998) Defining affinity with the GABAA receptor. *J Neurosci,* 18, 8590-8604.

Jones, M. V. and Westbrook, G. L. (1995) Desensitized states prolong GABAA channel responses to brief agonist pulses. *Neuron,* 15, 181-191.

Kam, K. and Nicoll, R. (2007) Excitatory synaptic transmission persists independently of the glutamate-glutamine cycle. *J Neurosci,* 27, 9192-9200.

Kapetanovic, I. M., Yonekawa, W. D. and Kupferberg, H. J. (1993) Time-related loss of glutamine from hippocampal slices and concomitant changes in neurotransmitter amino acids. *Journal of Neurochemistry,* 61, 865-872.

Kash, S. F., Johnson, R. S., Tecott, L. H., Noebels, J. L., Mayfield, R. D., Hanahan, D. and Baekkeskov, S. (1997) Epilepsy in mice deficient in the 65-kDa isoform of glutamic acid decarboxylase. *Proceedings of the National Academy of Sciences of the United States of America,* 94, 14060-14065.

Klausberger, T. and Somogyi, P. (2008) Neuronal diversity and temporal dynamics: the unity of hippocampal circuit operations. *Science, New York, N.Y,* 321, 53-57.

Kullmann, D. M. and Lamsa, K. P. (2007) Long-term synaptic plasticity in hippocampal interneurons. *Nature Reviews,* 8, 687-699.

Kvamme, E., Nissen-Meyer, L. S., Roberg, B. A. and Torgner, I. A. (2008) Novel form of phosphate activated glutaminase in cultured astrocytes and human neuroblastoma cells, PAG in brain pathology and localization in the mitochondria. *Neurochem Res,* 33, 1341-1345.

Kvamme, E. and Olsen, B. E. (1980) Substrate mediated regulation of phosphate-activated glutaminase in nervous tissue. *Brain Res,* 181, 228-233.

Kvamme, E., Torgner, I. A. and Roberg, B. (2001) Kinetics and localization of brain phosphate activated glutaminase. *Journal of Neuroscience Research,* 66, 951-958.

Laake, J. H., Slyngstad, T. A., Haug, F. M. and Ottersen, O. P. (1995) Glutamine from glial cells is essential for the maintenance of the nerve terminal pool of glutamate: immunogold evidence from hippocampal slice cultures. *Journal of Neurochemistry,* 65, 871-881.

Laake, J. H., Takumi, Y., Eidet, J., Torgner, I. A., Roberg, B., Kvamme, E. and Ottersen, O. P. (1999) Postembedding immunogold labelling reveals subcellular localization and pathway-specific enrichment of phosphate activated glutaminase in rat cerebellum. *Neuroscience,* 88, 1137-1151.

Lamsa, K., Heeroma, J. H. and Kullmann, D. M. (2005) Hebbian LTP in feed-forward inhibitory interneurons and the temporal fidelity of input discrimination. *Nature Neuroscience,* 8, 916-924.

Lang, U. and Frotscher, M. (1990) Postnatal development of nonpyramidal neurons in the rat hippocampus (areas CA1 and CA3): a combined Golgi/electron microscope study. *Anat Embryol (Berl),* 181, 533-545.

Laurie, D. J., Wisden, W. and Seeburg, P. H. (1992) The distribution of thirteen GABAA receptor subunit mRNAs in the rat brain. III. Embryonic and postnatal development. *J Neurosci,* 12, 4151-4172.

Lehre, K. P. and Danbolt, N. C. (1998) The number of glutamate transporter subtype molecules at glutamatergic synapses: chemical and stereological quantification in young adult rat brain. *J Neurosci,* 18, 8751-8757.

Leinekugel, X., Tseeb, V., Ben-Ari, Y. and Bregestovski, P. (1995) Synaptic GABAA activation induces Ca2+ rise in pyramidal cells and interneurons from rat neonatal hippocampal slices. *The Journal of Physiology,* 487 (Pt 2), 319-329.

Lerma, J., Herranz, A. S., Herreras, O., Abraira, V. and Martin del Rio, R. (1986) In vivo determination of extracellular concentration of amino acids in the rat hippocampus. A method based on brain dialysis and computerized analysis. *Brain Res,* 384, 145-155.

Liang, S. L., Carlson, G. C. and Coulter, D. A. (2006) Dynamic regulation of synaptic GABA release by the glutamate-glutamine cycle in hippocampal area CA1. *J Neurosci,* 26, 8537-8548.

Mackenzie, B. and Erickson, J. D. (2004) Sodium-coupled neutral amino acid (System N/A) transporters of the SLC38 gene family. *Pflugers Arch,* 447, 784-795.

Mackenzie, B., Schafer, M. K., Erickson, J. D., Hediger, M. A., Weihe, E. and Varoqui, H. (2003) Functional properties and cellular distribution of the system A glutamine transporter SNAT1 support specialized roles in central neurons. *The Journal of Biological Chemistry,* 278, 23720-23730.

Malenka, R. C. and Nicoll, R. A. (1997) Silent synapses speak up. *Neuron,* 19, 473-476.

Martinez-Hernandez, A., Bell, K. P. and Norenberg, M. D. (1977) Glutamine synthetase: glial localization in brain. *Science (New York, N.Y),* 195, 1356-1358.

Marty, S., Berninger, B., Carroll, P. and Thoenen, H. (1996) GABAergic stimulation regulates the phenotype of hippocampal interneurons through the regulation of brain-derived neurotrophic factor. *Neuron,* 16, 565-570.

Masson, J., Darmon, M., Conjard, A. et al. (2006) Mice lacking brain/kidney phosphate-activated glutaminase have impaired glutamatergic synaptic transmission, altered breathing, disorganized goal-directed behavior and die shortly after birth. *J Neurosci,* 26, 4660-4671.

Mathews, G. C. and Diamond, J. S. (2003) Neuronal glutamate uptake Contributes to GABA synthesis and inhibitory synaptic strength. *J Neurosci,* 23, 2040-2048.

McCormick, D. A. and Bal, T. (1997) Sleep and arousal: thalamocortical mechanisms. *Annu Rev Neurosci,* 20, 185-215.

McGale, E. H., Pye, I. F., Stonier, C., Hutchinson, E. C. and Aber, G. M. (1977) Studies of the inter-relationship between cerebrospinal fluid and plasma amino acid concentrations in normal individuals. *Journal of Neurochemistry,* 29, 291-297.

Megias, M., Emri, Z., Freund, T. F. and Gulyas, A. I. (2001) Total number and distribution of inhibitory and excitatory synapses on hippocampal CA1 pyramidal cells. *Neuroscience,* 102, 527-540.

Meisler, M. H. and Kearney, J. A. (2005) Sodium channel mutations in epilepsy and other neurological disorders. *J Clin Invest,* 115, 2010-2017.

Meldrum, B. S., Swan, J. H., Ottersen, O. P. and Storm-Mathisen, J. (1987) Redistribution of transmitter amino acids in rat hippocampus and cerebellum during seizures induced by L-allylglycine and bicuculline: an immunocytochemical study with antisera against conjugated GABA, glutamate and aspartate. *Neuroscience,* 22, 17-27.

Melikian, H. E. (2004) Neurotransmitter transporter trafficking: endocytosis, recycling, and regulation. *Pharmacol Ther,* 104, 17-27.

Melone, M., Quagliano, F., Barbaresi, P., Varoqui, H., Erickson, J. D. and Conti, F. (2004) Localization of the glutamine transporter SNAT1 in rat cerebral cortex and neighboring structures, with a note on its localization in human cortex. *Cereb Cortex,* 14, 562-574.

Melone, M., Varoqui, H., Erickson, J. D. and Conti, F. (2006) Localization of the Na(+)-coupled neutral amino acid transporter 2 in the cerebral cortex. *Neuroscience,* 140, 281-292.

Miles, R., Toth, K., Gulyas, A. I., Hajos, N. and Freund, T. F. (1996) Differences between somatic and dendritic inhibition in the hippocampus. *Neuron,* 16, 815-823.

Munoz, M. D., Herreras, O., Herranz, A. S., Solis, J. M., Martin del Rio, R. and Lerma, J. (1987) Effects of dihydrokainic acid on extracellular amino acids and neuronal excitability in the in vivo rat hippocampus. *Neuropharmacology,* 26, 1-8.

Neves, G., Cooke, S. F. and Bliss, T. V. (2008) Synaptic plasticity, memory and the hippocampus: a neural network approach to causality. *Nature Reviews,* 9, 65-75.

Norenberg, M. D. and Martinez-Hernandez, A. (1979) Fine structural localization of glutamine synthetase in astrocytes of rat brain. *Brain Res,* 161, 303-310.

Paulsen, R. E. and Fonnum, F. (1989) Role of glial cells for the basal and Ca2+-dependent K+-evoked release of transmitter amino acids investigated by microdialysis. *Journal of Neurochemistry,* 52, 1823-1829.

Paulsen, R. E., Odden, E. and Fonnum, F. (1988) Importance of glutamine for gamma-aminobutyric acid synthesis in rat neostriatum in vivo. *Journal of Neurochemistry,* 51, 1294-1299.

Petroff, O. A., Rothman, D. L., Behar, K. L. and Mattson, R. H. (1996) Low brain GABA level is associated with poor seizure control. *Annals of Neurology,* 40, 908-911.

Pinault, D. (2004) The thalamic reticular nucleus: structure, function and concept. *Brain Res Brain Res Rev,* 46, 1-31.

Proper, E. A., Hoogland, G., Kappen, S. M. et al. (2002) Distribution of glutamate transporters in the hippocampus of patients with pharmaco-resistant temporal lobe epilepsy. *Brain,* 125, 32-43.

Rae, C., Hare, N., Bubb, W. A., McEwan, S. R., Broer, A., McQuillan, J. A., Balcar, V. J., Conigrave, A. D. and Broer, S. (2003) Inhibition of glutamine transport depletes glutamate and GABA neurotransmitter pools: further evidence for metabolic compartmentation. *Journal of Neurochemistry,* 85, 503-514.

Rosenmund, C. and Stevens, C. F. (1996) Definition of the readily releasable pool of vesicles at hippocampal synapses. *Neuron,* 16, 1197-1207.

Ryan, T. A., Reuter, H. and Smith, S. J. (1997) Optical detection of a quantal presynaptic membrane turnover. *Nature,* 388, 478-482.

Schousboe, A., Larsson, O. M., Wood, J. D. and Krogsgaard-Larsen, P. (1983) Transport and metabolism of gamma-aminobutyric acid in neurons and glia: implications for epilepsy. *Epilepsia,* 24, 531-538.

Sepkuty, J. P., Cohen, A. S., Eccles, C., Rafiq, A., Behar, K., Ganel, R., Coulter, D. A. and Rothstein, J. D. (2002) A neuronal glutamate transporter contributes to neurotransmitter GABA synthesis and epilepsy. *J Neurosci,* 22, 6372-6379.

Seress, L. and Ribak, C. E. (1988) The development of GABAergic neurons in the rat hippocampal formation. An immunocytochemical study. *Brain Research,* 44, 197-209.

Soghomonian, J. J. and Martin, D. L. (1998) Two isoforms of glutamate decarboxylase: why? *Trends in Pharmacological Sciences,* 19, 500-505.

Solbu, T. T., Bjorkmo, M., Berghuis, P., Harkany, T. and Chaudhry, F. A. SAT1, A Glutamine Transporter, is Preferentially Expressed in GABAergic Neurons. *Front Neuroanat,* 4, 1.

Somogyi, P. and Klausberger, T. (2005) Defined types of cortical interneurone structure space and spike timing in the hippocampus. *The Journal of Physiology,* 562, 9-26.

Somwar, R., Kim, D. Y., Sweeney, G., Huang, C., Niu, W., Lador, C., Ramlal, T. and Klip, A. (2001) GLUT4 translocation precedes the stimulation of glucose uptake by insulin in muscle cells: potential activation of GLUT4 via p38 mitogen-activated protein kinase. *The Biochemical Journal,* 359, 639-649.

Spruston, N. (2008) Pyramidal neurons: dendritic structure and synaptic integration. *Nature Reviews,* 9, 206-221.

Stafford, M. M., Brown, M. N., Mishra, P., Stanwood, G. D. and Mathews, G. C. (2010) Glutamate spillover augments GABA synthesis and release from axodendritic synapses in rat hippocampus. *Hippocampus*, 20, 134-144.

Szerb, J. C. and O'Regan, P. A. (1984) Glutamine enhances glutamate release in preference to gamma-aminobutyrate release in hippocampal slices. *Can J Physiol Pharmacol,* 62, 919-923.

Tani, H., Bandrowski, A. E., Parada, I., Wynn, M., Huguenard, J. R., Prince, D. A. and Reimer, R. J. (2007) Modulation of epileptiform activity by glutamine and system A transport in a model of post-traumatic epilepsy. *Neurobiology of Disease,* 25, 230-238.

Traynelis, S. F. and Dingledine, R. (1988) Potassium-induced spontaneous electrographic seizures in the rat hippocampal slice. *Journal of Neurophysiology,* 59, 259-276.

Tyzio, R., Represa, A., Jorquera, I., Ben-Ari, Y., Gozlan, H. and Aniksztejn, L. (1999) The establishment of GABAergic and glutamatergic synapses on CA1 pyramidal neurons is sequential and correlates with the development of the apical dendrite. *J Neurosci,* 19, 10372-10382.

van den Berg, C. J. and Garfinkel, D. (1971) A stimulation study of brain compartments. Metabolism of glutamate and related substances in mouse brain. *The Biochemical Journal,* 123, 211-218.

Varoqui, H., Zhu, H., Yao, D., Ming, H. and Erickson, J. D. (2000) Cloning and functional identification of a neuronal glutamine transporter. *The Journal of Biological chemistry,* 275, 4049-4054.

Vitellaro-Zuccarello, L., Calvaresi, N. and De Biasi, S. (2003) Expression of GABA transporters, GAT-1 and GAT-3, in the cerebral cortex and thalamus of the rat during postnatal development. *Cell Tissue Res,* 313, 245-257.

Waagepetersen, H. S., Sonnewald, U., Larsson, O. M. and Schousboe, A. (1999) Synthesis of vesicular GABA from glutamine involves TCA cycle metabolism in neocortical neurons. *Journal of Neuroscience Research,* 57, 342-349.

Wang, D. D. and Kriegstein, A. R. (2008) GABA regulates excitatory synapse formation in the neocortex via NMDA receptor activation. *J Neurosci,* 28, 5547-5558.

Wang, D. D. and Kriegstein, A. R. (2009) Defining the role of GABA in cortical development. *The Journal of Physiology,* 587, 1873-1879.

Weiss, M. D., Derazi, S., Rossignol, C., Varoqui, H., Erickson, J. D., Kilberg, M. S. and Anderson, K. J. (2003) Ontogeny of the neutral amino acid transporter SAT1/ATA1 in rat brain. *Brain Research,* 143, 151-159.

Wu, H., Jin, Y., Buddhala, C. et al. (2007) Role of glutamate decarboxylase (GAD) isoform, GAD65, in GABA synthesis and transport into synaptic vesicles-Evidence from GAD65-knockout mice studies. *Brain Res,* 1154, 80-83.

Yao, D., Mackenzie, B., Ming, H., Varoqui, H., Zhu, H., Hediger, M. A. and Erickson, J. D. (2000) A novel system A isoform mediating Na+/neutral amino acid cotransport. *The Journal of Biological Chemistry,* 275, 22790-22797.