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GLUTAMATE DYSREGULATION AND HIPPOCAMPAL DYSFUNCTION IN
EPILEPTOGENESIS

THESIS

A thesis submitted in partial fulfillment of the
requirements for the Master of Science in the
College of Medicine at the
University of Kentucky

By

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Lexington, Kentucky

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Lexington, Kentucky

2013

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ABSTRACT OF THESIS

GLUTAMATE DYSREGULATION AND HIPPOCAMPAL DYSFUNCTION IN EPILEPTOGENESIS

Epileptogenesis is the complex process of the brain developing epileptic activity. Due to the role of glutamate and the hippocampus in synaptic plasticity a dysregulation in glutamate neurotransmission and hippocampal dysfunction are implicated in the process of epileptogenesis. However, the exact causal factors that promote epileptogenesis are unknown.

We study presynaptic proteins that regulate glutamate neurotransmission and their role in epileptogenesis. The presynaptic protein, tomosyn, is believed to be a negative regulator of glutamate neurotransmission; however, no one has studied the effects of this protein on glutamate transmission *in vivo*. Furthermore, evidence suggests that mice lacking tomosyn have a kindling phenotype. Thus, *in vivo* glutamate recordings in mice lacking tomosyn have the potential to elucidate the exact role of tomosyn in glutamate neurotransmission and its potential relationship to epileptogenesis.

Here we used biosensors to measure glutamate in the dentate gyrus (DG), CA3, and CA1 of the hippocampus in tomosyn wild-type (Tom^{+/+}), heterozygous (Tom^{+/-}), and knock out (Tom^{-/-}) mice. We found that, in the DG, that glutamate release increases as tomosyn expression decreases across genotype. This suggests that tomosyn dysregulation in the DG leads to an increase in glutamate release, which may explain why these mice have an epileptogenic phenotype.

KEYWORDS: Electrochemistry, Epileptogenesis, Glutamate, Hippocampus,
Tomosyn

Seth R. Batten

July 25, 2013

GLUTAMATE DYSREGULATION AND HIPPOCAMPAL DYSFUNCTION IN
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Dedicated to Granddad and Nanny

You always told me an education was one of
the best things I could ever obtain–
you were right.

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Raymond Carver, a true master of the short story, had this to say about writing in his essay *On Writing*:

“It is possible to write a line of seemingly innocuous dialogue and have it send a chill along the reader’s spine– the source of artistic delight, as Nabokov would have it. That’s the kind of writing that most interests me. I hate sloppy or haphazard writing whether it flies under the banner of experimentation or else is just clumsily rendered realism. In Isaac Babel’s wonderful short story, ‘Guy de Maupassant’, the narrator has this to say about the writing of fiction: ‘No iron can pierce the heart with such force as a period put just at the right place.’”

I also feel this way about writing albeit it is likely hard to tell from the series of technical sounding run-on sentences you are about to endure. Nevertheless, in my head, “innocuous dialogue that sends a chill down the reader’s spine” is how I think I write (we all have delusions of grandeur). I have read a number of thesis acknowledgement sections in my time in graduate school and all of them tend to lean towards ‘Academy Award-esk’ presentations. While I do not necessarily disagree with this type of writing I know that if I try to attempt it I will undoubtedly say something silly or offensive in my aimless rambling. Thus, I am trying to avoid “sloppy and haphazard writing” by attempting to be brief.

We as scientists (or students of), in my opinion, have one of the greatest jobs in the world. It is our duty to simply be enamored by nature and to try and quantify it and understand it. Much like a poet or author we have the privilege of gazing at a puddle of water in the street or at the way the leaves on a tree flutter, from dark to light, as a storm rolls in. However, unlike a poet or author who usually only encompass the intellectual capacity to say, “Why?” we, as scientists, have the ability to know how to test it– to *learn* why. This is the true beauty of science. I feel truly blessed to be able to come to work every day and watch

lines on a screen accurately represent what neurotransmitters are doing in the brain. Every day I get to observe the exciting tango of molecular probabilities—the probabilities that create the human condition. There is no better thing to witness.

In our scientific endeavors we have been together— when the ship was sailing and when it was sinking— working tirelessly to move forward. I truly believe that you are only as good as the company you keep. I am better and smarter for knowing and working with you all. Thank you for taking me in, thank you for teaching me, and most of all thank you for putting up with me. As friends and colleagues go, I hope we have many more years together. Kurt Vonnegut once said, “A purpose of human life, no matter who is controlling it, is to love whoever is around to be loved.” On my best and even my worst days I attempt to live by this. I love you all. Thank you for everything. I hope I put all my periods “just at the right place”.

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Chapter One: Introduction

L-Glutamate is the neurotransmitter necessary for proper cognitive functioning and memory formation (Platt, 2007). However, dysregulation of glutamate is thought to underlie several neurological and psychiatric diseases (Meldrum, 2000). Specifically, hippocampal glutamate dysregulation seems to be a factor in several diseases (Rupsingh et al., 2011; Billa et al., 2010; Scheyer, 1998).

The hippocampus is the brain area most associated with learning and memory (Deng et al., 2010). In the hippocampus glutamate transmitter and receptor dynamics, specifically through long-term potentiation (LTP) and long-term depression (LTD), are considered to be the underlying mechanisms that are thought to promote memory formation and synaptic plasticity (Lüscher & Malenka, 2012). However, aberrant synaptic plasticity can occur which can promote diseases such as epilepsy (Scheyer, 1998).

Epileptogenesis is a complex process involving molecular, cellular, and neural network changes that result in dysregulated synaptic activity (Bertram, 2007). Furthermore, some have suggested that the process of epileptogenesis is similar to the molecular changes that occur in the formation of normal long-term memories (Hannesson & Corcoran, 2000). Since glutamate neurotransmission and the hippocampus are essential in memory formation and synaptic plasticity dysfunctions of these systems may promote epileptogenesis (Morris et al., 2000; Wasterlain et al., 2000). We have focused on studying glutamate dynamics in the hippocampus by specifically examining changes in presynaptic proteins that regulate glutamate release and their effect on epileptogenesis (Matveeva et al., 2011a; Matveeva et al., 2011b; Matveeva et al., 2008; Matveeva et al., 2007; Matveeva et al., 2003). To this end, we have used the kindling model of epileptogenesis to assess these molecular and neurotransmitter changes.

Previously our group has shown that kindling in rats promotes asymmetrical accumulation of one of the major components of the secretory machinery, the 7 svedberg SNAP (soluble N-ethylmaleimide sensitive factor

[NSF] attachment protein) receptor (7S SNARE) complex (7SC) (Matveeva et al., 2008; Matveeva et al., 2007; Matveeva et al., 2003). Minimally, this complex is a heterotrimer composed of the membrane-bound t-SNAREs, syntaxin and synaptosomal-associated protein 25 (SNAP-25), and the vesicle-bound v-SNARE, synaptobrevin/vesicle-associated membrane protein 2 (VAMP2) (Jahn & Scheller, 2006). Furthermore, we have shown that synaptic vesicle protein (SV2) and NSF, regulators of the 7SC, show long-term alterations in the ipsilateral hippocampus in the CA1 and DG following kindling (Matveeva et al., 2008). Also, roughly a 50% knock down of VAMP2 in mice created a kindling resistant phenotype (requiring more current and stimulations to reach a fully kindled state) and caused a decrease in KCl-evoked glutamate release in the DG and CA3 of the hippocampal formation (Matveeva et al., 2011b). These results suggest that proteins involved in glutamate release and regulation may have a role in epileptogenesis.

Tomosyn is a presynaptic protein that negatively regulates glutamate release (Sakisaka et al., 2008). Tomosyn is thought to inhibit neurotransmitter release primarily by sequestering syntaxin and thus inhibiting 7SC formation (Asher et al., 2009) (Figure 1.1). Furthermore, tomosyn is preferentially expressed in the hippocampus mainly in glutamatergic synapses (Brakak et al., 2010). Evidence from hippocampal mossy fibers has shown that neurons lacking tomosyn have an increased probability of neurotransmitter release as measured electrophysiologically looking at excitatory post-synaptic potentials (EPSPs) (Sakisaka et al., 2008). Building on those results, we have shown that tomosyn knockout mice exhibit a kindling phenotype requiring fewer stimulations to reach a Racine stage 5 seizure (Figure 1.2). Taken together, the above evidence suggests that tomosyn dysregulation may cause increased glutamate release and promote epileptogenesis.

No group has yet measured glutamate release *in vivo* in tomosyn knockout mice to assess whether changes in tomosyn expression actually affect glutamate release in an intact biological system. Here we measured glutamate in subregions of the hippocampus (dentate gyrus [DG], CA3, and CA1) using

biosensors selective for glutamate in tomosyn wild type (Tom^{+/+}), tomosyn heterozygous (Tom^{+/-}), and tomosyn knockout (Tom^{-/-}) mice. Using biosensors with a high temporal and spatial resolution this thesis presents data on glutamate measurements from mice lacking tomosyn. In chapter one, the glutamate system, hippocampus, and epileptogenesis are discussed, in chapter two the methodology used is discussed, and in chapter three the data are presented and discussed.

Glutamate System Dynamics

Glutamate, like most neurotransmitters, maintains homeostatic tone by tightly regulating release and uptake as well as the receptors and transporters associated with these processes. Here the specifics of the glutamate system are discussed starting with release then moving on to discuss transporters and receptors and how these dynamics are related to epileptogenesis.

Synthesis & Release

Glutamine and α -ketoglutarate are the two precursors in glutamate synthesis (Tapiero et al., 2002). Glutamine and α -ketoglutarate are taken up by neurons in a Na⁺ dependent fashion; glutamate is synthesized from glutamine via the enzyme glutaminase and from α -ketoglutarate via a transamination reaction (Anderson & Swanson, 2000; Daikhin & Yudkoff, 2000). Glutamate is then taken up by vesicular glutamate transporters (VGLUTs) in an energy dependent fashion and packaged into vesicles (Fonnum et al., 1998) (Figure 1.3). After packaging, glutamate is released in a Ca²⁺ dependent fashion into the synaptic cleft (Meldrum, 2000; Turner, 1998). Once released, glutamate either: (a) binds to pre and post-synaptic glutamate receptors, (b) is actively taken up by glia and synthesized back into glutamine via glutamine synthase, (c) actively transported by presynaptic neurons and repackaged, or (d) diffuses away from the synapse (Anderson & Swanson, 2000; Attwell, 2000; Daikhin & Yudkoff, 2000).

Transporters

Five glutamate transporters have been found in the mammalian central nervous system (CNS) (Meldrum, 2000). Of these five, two are expressed in glial

cells and are responsible for 90% of glutamate uptake (excitatory amino acid transporter 1 [EAAT1; referred to as GLAST1 in rats] and EAAT2 [referred to as GLT1 in rats]) and three post-synaptically in neurons (EAAT3 [referred to as EAAC1 in rats; cortical neurons], EAAT4 [cerebellar Purkinje neurons], EAAT 5 [retina neurons]) (Iverson et al., 2009; Danbolt et al., 1998; Seal & Amara, 1999) (Figure 1.4). EAAT1-5 all transport glutamate in a Na⁺ dependent fashion (Kataoka et al., 1997). Interestingly, EAAT3-5 seem to be linked to Cl⁻ channels as well as being sodium dependent; when glutamate binds to the postsynaptic transporters Cl⁻ channels also open and decreases synaptic activity via hyperpolarization (Levy et al., 1998). This is thought to be a negative-feedback system for glutamate release (Levy et al., 1998).

These transporters are enantioselective (D-Aspartate [D-Asp], L-Aspartate [L-Asp], and L-glutamate are all substrates whereas D-glutamate is not) with affinities that range from K_m values from 10-100 μ M with the exception of EAAT4, which has a K_m value of approximately 2 μ M (note this affinity is much higher than that seen by VGLUTs with K_m values approximately equal to 1 mM) (Iverson et al., 2009; Meldrum, 2000; Danbolt et al., 1998). Once glutamate is taken up by glia and/or neurons it is metabolized and recycled (see 'Synthesis & Release' section). Now that glutamate synthesis, release, and transporters have been discussed, next the receptors to which neuronally-released glutamate can bind to will be reviewed.

Glutamate Ionotropic Receptors

There are three classes of ionotropic receptors: *N*-methyl-*D*-aspartate (NMDA), α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), and kainic acid (Iverson et al., 2009; Meldrum, 2000). Each was identified and defined first by their pharmacology and later by their molecular biology (Meldrum, 2000; Tzschentke, 2002). Here all three are discussed in detail because of their suggested dysfunction in epileptogenesis and epilepsy (Higuchi et al., 2000; Musshoff et al., 2000).

The NMDA Receptor

The NMDA receptors (NMDARs) are ligand and voltage-gated, post-synaptic ion channels that are permeable to Na^+ , K^+ , and Ca^{2+} (Kew & Kemp, 2005; Madden, 2002) (Figure 1.4). The receptor has several modulatory binding sites: (a) glutamate binding site that binds not only transmitter but related agonists as well (within this area it has been suggested that one part of this site has a preference for agonist while the other a preference for antagonist), (b) a glycine binding site; glycine is a necessary cofactor for glutamate activation of the NMDAR however recent evidence suggests that this site may bind D-serine, which is not at saturating levels in the brain like glycine, (c) a site inside the receptor that binds phencyclidine (PCP) and other noncompetitive antagonists, (d) a voltage-dependent Mg^{2+} binding site inside the receptor, (e) an inhibitory cation site located near the mouth of the channel that binds Zn^{2+} causing a voltage-independent block, and (f) a polyamine regulatory site whose activation by spermine and spermidine can enhance NMDAR activity at lower concentrations but inhibit the receptor at higher concentrations (Iverson et al., 2009; Mayer, 2005; Kew et al., 2000; Madden, 2002; Anson et al., 1998).

The above regulatory sites are found on the different complexes of the NMDARs (Kew et al., 2000; Anson et al., 1998). The NMDARs are primarily composed of two families of subunits: NR1 subunit (represented by one gene) and the NR2 subunit (represented by four genes [NR2A-NR2D]) (Kew & Kemp, 2005; Meldrum, 2000). There is also some evidence that NR3A and NR3B subunits also exist, and while they seem to decrease the NMDARs' Ca^{2+} permeability, the physiological significance of these subunits are not well understood (Matsuda et al., 2002; Sasaki et al., 2002; Nishi et al., 2001). There is some evidence that NR1 homomeric NMDARs can exist although they do not seem to pass ions very readily and their ion conductance increases approximately 100-fold when coexpressed with NR2 subunits (Nestler et al., 2009). Recent evidence suggests that this increase in conductance is due to the glutamate binding site being located on the NR2 subunit; thus, it is now believed that most functional NMDARs are composed of heteromeric complexes of NR1

and NR2 subunits (Kew & Kemp, 2005). All of the regulatory sites found on these difference subunits work together to modulate the function of the receptor.

The various regulatory sites found on the subunits on the NMDAR has prompted the appellation 'coincident receptor' due its necessity for two coagonists (glutamate and glycine/D-serine) as well as the voltage-dependent removal of the Mg^{2+} ion for NMDAR function (Nestler et al., 2009; Madden, 2002; Kew et al., 2000; Anson et al., 1998). Along with this, the NMDARs' permeability to Ca^{2+} has drawn much attention due to the many modulatory roles Ca^{2+} performs inside neurons (Lüscher & Malenka, 2012). Thus, the NMDARs role as a 'coincident detector' as well as its permeability to Ca^{2+} seems to be the driving force for NMDARs in synaptic plasticity, perhaps even for the aberrant synaptic plasticity that may be involved in epileptogenesis (Morris et al., 2000; Wasterlain et al., 2000).

The AMPA Receptor

AMPA receptors (AMPA receptors) are ligand-gated, post-synaptic ion channels with two glutamate binding sites that, when bound to glutamate, allow the influx of Na^+ and Ca^{2+} (subtype specific; see below) and the efflux of K^+ (Dingledine et al., 1999) (Figure 1.4). AMPARs have a lower affinity for glutamate compared to NMDA receptors; however, AMPA receptors do have faster kinetics and are responsible for the quicker initial component of EPSPs (Dingledine et al., 1999).

AMPA receptors exist as heterotetramers consisting of various combinations of subunits termed GluR1-4 (Rosenmund et al., 1998). The majority of AMPARs contain GluR2 subunits and can only pass Na^+ and K^+ ions; however, some AMPARs lack the GluR2 subunit and are permeable to Ca^{2+} (Bowie & Mayer, 1995). GluR2 lacking receptors are able to pass Ca^{2+} ions due to a difference in the glutamine/arginine site (Q/R site) on the GluR2 subunit; the GluR1,3,4 subunits contain a glutamine at the Q/R site whereas on the GluR2 subunit this site contains an arginine which repels the Ca^{2+} ions thus preventing them from entering neurons (Seeburg & Hartner, 2003; Dingledine et al., 1999). AMPARs can also exist in different splice variants termed Flip and Flop that influence the rate of desensitization and the efficacy of certain allosteric modulators (Kew &

Kemp, 2005). Different cells in various brain regions can also express different subtypes and splice variants of AMPARs.

Evidence suggests that, in the hippocampus, GluR2 lacking AMPARs are predominantly found on inhibitory interneurons whereas GluR2 containing AMPARs are found predominantly on pyramidal neurons (Mahanty & Sah, 1998). Further, there is evidence that Ca^{2+} entering through GluR2 lacking receptors may prompt migration of GluR2 containing receptors to the cell membrane (Liu & Cull-Candy, 2002). Taken together, this evidence suggests not only another level of synaptic plasticity but also a potential glutamatergic/AMPA self-regulatory mechanism. Further, differential expression of GluR1-4 containing AMPARs are seen in epileptogenesis suggesting that the expression of these receptors changes in epilepsy, which may initiate and/or promote this disease (Higuchi et al., 2000). A similar trend is seen with kainate receptors.

Kainate Receptor

The kainate receptor is a ligand-gated ion channel located both pre and post-synaptically (Lerma, 2003). When activated by glutamate, presynaptic kainate receptors can facilitate or inhibit neurotransmission; post-synaptically kainate receptor stimulation causes slow EPSPs (Cossart et al., 2002; Lauri et al., 2001; Kidd & Isaac, 1999). The receptor contains two glutamate binding sites that must both be bound by glutamate in order for the ion channel to open and allow Na^+ and Ca^{2+} (depending on subunit composition) influx and K^+ efflux (Pinaheiro & Mulle, 2006; Sommer et al., 1991) (Figure 1.4).

Kainate receptors are tetrameric complexes of GluR5-7 and KA1-2 subunits (Bleakman et al., 2002). Homotetrameric complexes of KA1-2 subunits do not create functional receptors; however, homotetrameric complexes of GluR5-7 subunits do create functional receptors (Alt et al., 2004; Gallyas et al., 2003; Bleakman et al., 2002). Further, heterotetrameric complexes of GluR5-7 and KA1-2 frequently occur and create functional kainate receptors as well (Bleakman et al., 2002).

GluR5-7 subunits can be alternately spliced to induce greater receptor variation and GluR5 and GluR6 subunits can be edited at the Q/R site to vary the

receptors permeability to Ca^{2+} (Dingledine et al., 1999; Schiffer et al., 1997; Herb et al., 1996). Kainate receptors are differentially expressed in the brain (Contractor et al., 2000). For example, there is evidence that GluR5 expressing kainate receptors are in high concentration in the temporal lobe (Rogawski et al., 2003). Further, these receptors can even be differentially expressed in different subregions of a given brain area such as in the hippocampus (Contractor et al., 2000). KA1 and KA2 containing receptors are found in DG mossy fibers where KA2 containing receptors are predominately post-synaptic; KA2-GluR5 heteromeric receptors seem to be expressed post-synaptically in pyramidal neurons in the CA3 (Jaskolski et al., 2005). Thus, with varying splice forms and differential CNS expression these receptors allow further complexity and modes for synaptic plasticity. Further, their dysfunction has been implicated in epileptogenesis (Putkonen et al., 2011). Ionotropic receptors are not the only receptors important in glutamate homeostasis nor are they the only ones implicated in epileptogenesis. Metabotropic receptors are also an important aspect of the glutamate system and their proper functioning are essential to glutamate homeostasis as well.

Metabotropic Glutamate Receptors

Metabotropic glutamate receptors (mGluRs) are seven *trans*-membrane spanning G-protein coupled receptors that signal to various second messenger systems and that, when activated by glutamate, have a slower more modulatory role than ionotropic receptors (Pin & Acher, 2002; Kunishima et al., 2000). There are currently eight subtypes of mGluRs separated into three groups according to their sequence homology, second messenger systems, and pharmacology (Kunishima et al., 2000; Dingledine et al., 1999). It is thought that a malfunction of these receptors may promote epileptogenesis by inducing excitotoxicity not only by binding to glutamate but also by not appropriately modulating other neuronal receptors (Sayin & Rutecki, 2003; Meldrum et al., 1999). Thus, their importance in normal and pathological functions is discussed here.

Group I mGluRs

This group of metabotropic receptors contains mGluR1 and mGluR5 (Niswender & Conn, 2010). These receptors are found post-synaptically and they both have excitatory actions on neurons (Niswender & Conn, 2010; Coutinho & Knopfel, 2002) (Figure 1.4). When bound by glutamate, mGluR1,5s work through second messenger systems via G_q proteins which stimulate phospholipase C (PLPC) to create/release inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG), both which work to release Ca^{2+} from intracellular stores to several effector proteins (Hermans & Challiss, 2001). DAG also stimulates protein kinase C (PKC) which can then stimulate several different downstream effectors as well (Hermans & Challiss, 2001). Activation of these receptors may also promote synaptic plasticity via LTP and LTD (Bellone et al., 2008; Kullmann & Lamsa, 2008). mGluR1,5s can also undergo alternate splicing thus increasing receptor variation and their effect on cellular function (Joly et al., 1995; Pin & Duvoisin, 1995).

Evidence suggests that mGluR1,5 may both promote epileptogenesis; however, mGluR5 seems to be more important in inducing this disease process (Bianchi et al., 2012). It has been shown that this epileptogenic effect is independent of ionotropic receptors and that the activation of these mGluR group I receptors causes long lasting enhancement in the responsiveness of hippocampal networks (Merlin, 1999). One such long lasting change seems to be the development of a voltage-dependent cationic current that promotes rapid neuronal firing and prolonged action potentials in the hippocampus (Chuang et al., 2005; Young et al., 2004). Thus, the malfunction of the mGluR group I receptors seems to have a role in epileptogenesis.

Group II mGluRs

Group II mGluRs comprise mGluR2 and mGluR3 (Niswender & Conn, 2010). These receptors are found both pre and post-synaptically and, when bound by glutamate, generally have an inhibitory effect on excitatory, inhibitory, and modulatory neurons (Niswender & Conn, 2010; Tamaru et al., 2001) (Figure 1.4). mGluRs2,3 have primarily a pre-terminal localization on presynaptic cells

and mGluR3 may also be present on glial cells (Ferraguti & Shigemoto, 2006; Tamaru et al., 2001). The presynaptic mGluRs2,3 can be activated by excess synaptic glutamate or glutamate release from astrocytes via the cysteine-glutamate transporter (Kalivas, 2009).

The receptors generally work through $G_{i/o}$ proteins which inhibit adenylyl cyclase (AC) and cAMP formation and releases $G_{\beta\gamma}$ which affects downstream signaling proteins as well as directly activates K^+ channels and inhibits voltage-sensitive Ca^{2+} channels (Pin & Duvoisin, 1995; Tanabe et al., 1992). The diversity of the mGluRs2,3 and their effects on neurons can also be increased by alternate splicing (Sartorius et al., 2006). There is evidence to suggest that dysfunctional mGluRs2,3 may be important in the process of epileptogenesis.

Kew et al. (2001) found that agonists binding at these receptors decreases EPSPs in hippocampal neurons suggesting that a decrease in receptor number or a malfunction resulting in reduced agonist binding may increase EPSPs in hippocampal neurons. It has been shown that mGluRs2,3 activation in the hippocampus decreases glutamate release and that stimulation of mGluR3 on glial cells may increase glutamate uptake (Mateo & Porter, 2007; Zhao et al., 2006). Further, activation of these receptors also increased the seizure threshold in kindled rodents (Attwell et al., 1998). Thus, these receptors work to decrease neuronal excitation and may suppress epileptogenesis however any malfunction in these receptors may promote epileptogenesis.

Group III mGluRs

The group III receptors consist of mGluRs4,7,8 and are primarily located presynaptically in the active zone of neurons (Niswender & Conn, 2010) (Figure 1.4). When bound by glutamate these receptors generally inhibit neurotransmitter release and due to location of these receptors in the active zone of neurons they have the ability to regulate cells via negative-feedback mechanisms (Niswender & Conn, 2010). Furthermore, mGluR7 has a lower affinity for glutamate compared to mGluRs4,8, thus it takes robust glutamate release to activate mGluR7 compared to others in this group (Schoepp et al., 1999).

mGluRs 4,7,8 are coupled to $G_{i/o}$ proteins which inhibit AC and cAMP formation and releases $G_{\beta\gamma}$ which affects downstream signaling proteins (Pin & Duvoisin, 1995). mGluRs7,8 can be alternately spliced but mGluR4 cannot be, thus more diversity is seen in mGluRs7,8 in contrast to mGluR4 receptors (Malherbe et al., 1999; Corti et al., 1998). Note that mGluR6 also fits into this group however this receptor subtype is only located on bipolar cells in the retina and seems to have no role in epileptogenesis in the hippocampus (Nicoletti et al., 2011).

Group III mGluRs generally seem to be antiepileptic: an increase in mGluR4 is seen in patients with a history of temporal lobe epilepsy whereas no such increase is seen in controls. This would suggest that a compensatory increase in mGluR4 is seen in those undergoing epileptogenesis (Lie et al., 2000). Further, there is evidence that mGluR group III agonists reduce seizures in rodents (Tizzano et al., 1995). Thus, the role of these receptors in epileptogenesis is similar to the group II mGluRs.

Many disruptions in the glutamate system may occur in epileptogenesis ranging from issues with glutamate release to receptor malfunctions. However, no single malfunction alone seems to drive epileptogenesis; instead it seems that a synergistic effect drives a vast network change in the hippocampus that promotes this disease. Thus, the hippocampus as a network and the changes that it incurs must be discussed to fully attempt to understand epileptogenesis.

The Hippocampus: A Focus on Structure & Function

The hippocampus is a region of the brain that is implicated in many important processes such as spatial learning and memory. Dysfunction of the hippocampus is thought to cause several disease states including epileptogenesis. Here the structure and function of the trisynaptic pathway of the hippocampus are discussed specifically in relation to the glutamate system and epileptogenesis.

The Trisynaptic Pathway: Structure & Connectivity

There are two major glutamatergic systems that project through the hippocampus: (1) the unidirectional trisynaptic pathway, (2) the entorhinal cortex direct projections to specific hippocampal subfields (Tamminga et al., 2012). The trisynaptic pathway consists of the entorhinal cortex (EC), the DG, CA3, and CA1 (Amaral & Witter, 1989). The hippocampus in general, and specifically the trisynaptic pathway, is an area with a high number of glutamatergic neurons and glutamate projections (Amaral & Witter, 1989).

The trisynaptic path starts in the EC and the projections travel through the perforant path (through layers II/III) and synapse on the proximal dendrites of the DG granule cell neurons. Mossy fiber projections then carry signals to synapse on the proximal dendrites of CA3 pyramidal neurons. Signals then travel through the Schaffer collaterals bilaterally to dendrites in the CA1. CA1 pyramidal neurons then project to the subiculum which projects back to the EC, layer IV (Tamminga et al., 2012) (Figure 1.5).

The mossy fiber connections from the DG to the CA3 are of particular interest in the trisynaptic loop; one mossy fiber axon from the granule cell layer in the DG can create a glutamatergic synapse with 8-15 excitatory pyramidal neurons and 18-35 GABAergic interneurons all with Ca^{2+} dependent release dynamics (Pelkey & McBain, 2008; Lawrence & McBain, 2003; Toth et al., 2000). Further, each mossy fiber connection on interneurons has a distinct anatomical, electrophysiological, and molecular profile suggesting that this system is tightly controlled and regulated (Tamminga et al., 2012).

Results from measuring EPSPs *in vitro* also suggest that DG basal activity has a feed-forward inhibitory function on the CA3 that seems to occur through two distinct mechanisms in mossy fiber-interneuron connections from the DG to the CA3: (1) through post-synaptic GluR2-lacking AMPA receptors, (2) through presynaptic mGluR7 metabotropic receptors (Lawrence & McBain, 2003). Both of these mechanisms work to inhibit and stabilize neuronal firing in the CA3 either through the firing of GABAergic interneurons (mechanism [1]) or through

feedback inhibition of glutamate release from mossy fibers onto GABAergic interneurons (mechanism [2]) (Lawrence & McBain, 2003).

Interestingly, when stimulating the DG granule neurons with high frequency stimulation, the nature of mossy fiber signaling changes in the CA3 such that LTD occurs at the mossy fiber-interneuron synapse thus diminishing inhibition while also causing LTP to occur at the mossy fiber-pyramidal synapse; both result in excitation of CA3 neurons (Lawrence & McBain, 2003). The direct projections of the EC are to the CA3 and CA1 subfields of the hippocampus (Tamminga et al., 2012). These connections seem to be functionally important due to the fact that they might regulate CA3 and CA1 output along with neurons in the trisynaptic loop (Andersen et al., 2007; Witter, 1993). Taken together these various connections suggest that the trisynaptic loop is highly regulated and can work to perform very complex and specific tasks. Further, it also suggests that if this regulation is lost complex pathological changes such as what are seen in epileptogenesis could occur.

Although, many of these studies are conducted in rats, these data should be considered highly relevant to humans since the hippocampal formation is a conserved brain region in mammals (Andersen et al., 2007).

The Hippocampus: Function

The most documented function of the hippocampus is as a hub for synaptic plasticity, spatial memory, and emotional memory (Squire, 1992; Scoville & Milner, 1957). Evidence for this comes not only from the fact that glutamate is the primary neurotransmitter released from neurons in this area but also that LTP and LTD readily occur here (Lawrence & McBain, 2003; Amaral & Witter, 1989). In addition, there is now evidence that neurons readily regenerate in the DG of the hippocampus further showing the robust synaptic changes that can happen in this brain area (Zhao et al., 2008).

All types of memory formation may not occur here; for example there is evidence that the hippocampus may be more prone to consolidate spatial, context, episodic, and certain types of emotional memories as opposed to other types of memories such as declarative (Bonne et al., 2008; Stote & Fanselow,

2004; Kim & Fanselow, 1992). There is recent evidence that different regions of the hippocampus store different types of memories; for example the dorsal hippocampus may store spatial memories whereas the ventral hippocampus may store primarily emotional memories (Fanselow & Dong, 2010). The hippocampus is also the brain region that works to first learn new information (Battaglia et al., 2011). Because of the vast degree of synaptic plasticity that can occur in this area, it is not surprising that there is evidence of pathological synaptic plasticity, likely due to glutamate dysregulation, which can promote disease states such as epileptogenesis (Leonard & Kirby, 2002; Tzschentke, 2002; Chapman, 2000).

Epileptogenesis: The Role of Glutamate & The Hippocampus

Epilepsy is a debilitating disease characterized by chronic seizures. Epileptogenesis is the process of the brain becoming epileptic. The exact mechanism that drives epileptogenesis and epilepsy is unknown however there is evidence to suggest that epileptogenesis may be due to glutamate dysregulation specifically in the hippocampus. Epileptogenesis and the role of glutamate and the hippocampus in this process are discussed here.

What is Epileptogenesis?

The generally accepted definition of epileptogenesis is the process of the brain acquiring an initial insult and secondarily undergoing a series of epileptic events until the first observable seizure occurs (Giblin & Blumenfeld, 2010; Rakhade & Jensen, 2009; Walker et al., 2002). However, there is a debate on the exact definition of epileptogenesis specifically in terms of when epileptogenesis starts and at what time it evolves to epilepsy (Sloviter & Bumanglag, 2012).

Dudek & Staley (2011) and Pitkänen et al. (2011) expand the definition of epileptogenesis to incorporate the never-ending evolution of changes in seizure frequency and the development of a refractory state. On the other hand, Sloviter & Bumanglag (2012) have defined epileptogenesis as the finite process that leads to the first of a series of spontaneous and recurring epileptiform events that disrupt behavior or thought processes in anyway, whether they are clinically

relevant or not. Furthermore, Sloviter & Bumanglag (2012) have proposed a secondary term coupled to epileptogenesis they term “epileptic maturation” to describe the all encompassing processes that happen after epileptogenesis and that influence the secondary changes in the clinical phenotype. Thus, Sloviter & Bumanglag (2012) use these two terms to discuss different processes in the development of epilepsy.

While the specificity of the definition given by Sloviter & Bumanglag (2012) is warranted and enticing, this thesis uses the process of the brain acquiring an initial insult and secondarily undergoing a series of epileptic events until the first observable seizure is observed as the working definition of epileptogenesis.

Kindling: A Model of Epileptogenesis

Kindling is a process whereby an electrode, implanted in a limbic structure in the brain, delivers an electrical stimulus that causes progressive and permanent intensification of epileptiform, after-discharge (AD) activity that results in generalized seizures after the same repeated subconvulsive electrical stimulation (Goddard et al., 1969). In other words, the same current strength and duration cause the animal to have a ‘worse’ seizure than before; a decrease in seizure threshold occurs (called kindling acquisition).

In rodents the progression of kindling has been well characterized electrographically and behaviorally (Racine, 1972). These different stages mimic human seizures, with Racine stages 1-2 simulating complex partial seizures and stages 3-5 mimicking secondary generalized seizures (Racine, 1972). Once a fully kindled state is achieved (defined as two consecutive Racine 5 seizures for any data presented in this thesis) spontaneous seizures may occur for the rest of the animal’s life especially if the animal receives repeated stimuli over consecutive months (Coulter et al., 2002; Racine, 1972). Furthermore, because it is easier to induce a seizure in a animal once it has reached a stage 5 seizure (see above), it is hypothesized that a permanent neurobiological change has taken place in the brain; what drives that change is currently unknown (McIntyre & Gilby, 2006). Several investigators have used the kindling model to study the neurobiological changes that the brain undergoes at different stages of

epileptogenesis (see Stables et al., 2003). However, this model is not without pitfalls.

Because electrical current is sent through the brain during kindling, some investigators believe this model may be a bit extreme or different compared to the 'natural' process of epileptogenesis that occurs in humans (Reisner, 2003). Thus, some arguments against using the kindling model are concerned with face validity (i.e. how well does kindling represent human epileptogenesis?). Another failing of the kindling model is that not all animals have spontaneous seizures when kindled; thus, researchers may be studying changes in the brain that are not relevant to spontaneous seizures if they do not select out only those animals that *do* spontaneously seize to perform their experiments on (Pitkänen & Halonen, 1998). Another issue that has been raised is that the implantation of the kindling electrode into the brain may be a major cause of kindling rather than the electrical stimulation; thus, it calls into question what causal factor one is attempting to model (i.e. epileptogenesis from traumatic insult vs. electrical insult) (Löscher, 2002). However, it should be noted that introduction of kindling electrodes seldom if ever lead to seizures and only a relatively narrowly defined current frequency and duration will produce the kindled seizure state (Löscher, 2002). Regardless, one has to keep in mind that the ambiguity created from a poorly defined model can make results difficult to interpret and translate (i.e. does a medication that works for epilepsy caused by traumatic brain injury also work for 'idiopathic' epilepsy?).

Despite these shortcomings, the similarity of kindled seizures to human seizures, the chronic nature of seizures in kindling, and the fact that pharmaceuticals that prevent kindling have been effective in the clinic suggests that kindling is accurate at modeling the stages of epileptogenesis (Löscher, 2002).

Epileptogenesis: Glutamate Dynamics & The Hippocampus

Epileptogenesis is a complex process involving molecular, cellular, and neural network changes that result in dysregulated synaptic activity (Bertram, 2007). A lot of these changes occur in the hippocampus and glutamate

dysregulation may be a driving factor (Matveeva et al., 2011a; Matveeva et al., 2011b; Frasca et al., 2011; Bertram, 2009; Platt, 2007; Dalby & Mody, 2001; Najm et al., 2001).

Evidence from several researchers suggests that there is an increase in glutamate excitatory transmission in the hippocampus of animals and humans undergoing epileptogenesis (Matveeva et al., 2011a; Matveeva et al., 2011b; Frasca et al., 2011; Bertram, 2009; Platt, 2007; Dalby & Mody, 2001; Najm et al., 2001). Further evidence shows that glutamate release and receptor activation is increased after kindling especially in the DG (Matveeva et al., 2011a; Dalby & Mody, 2001). GLT-1-lacking mice are also seizure prone further supporting the role for glutamate excitotoxicity in epileptogenesis (Watanabe et al., 1999). There is also an increase in the pool of ready-release glutamate at the mossy fiber-pyramidal cell synapse in the CA3 as well as in DG neurons suggesting an alteration in release probability (van der Hel et al., 2009; Goussakov et al., 2000). More glutamate synapses, likely through mossy fiber sprouting, may appear during epileptogenesis as well which could cause increased levels of extracellular glutamate and glutamate system dysregulation (van der Hel et al., 2009).

The number of NMDA receptors present in neuronal cell membranes appears to increase during epileptogenesis (Musshoff et al., 2000). The increased presence of NMDA receptors in neuronal membranes suggests a role for synaptic plasticity in epileptogenesis; particularly since MK-801, an NMDA antagonist, has antiepileptic effects during kindling but has no effect on acute seizures (Morris et al., 2000; Wasterlain et al., 2000). Finally, evidence suggests that ifenprodil, a NR2B containing NMDA antagonist, reduces pyramidal neuron loss in the hippocampus during epileptogenesis (Frasca et al., 2011). Taken together, these data suggest that NMDA receptors have an early role in disease formation but do not have much of a role in disease maintenance or seizure activity per se (Dalby & Mody, 2001; Wasterlain et al., 2000). There is evidence that GluR2-lacking AMPA receptors are increased during epileptogenesis suggesting that there may be more Ca^{2+} influx into neurons which could cause

excitotoxicity and cell death as well as synaptic changes due to alterations in second messenger signaling (Higuchi et al., 2000; Prince et al., 2000). GluR6,7 containing kainate receptors may also be decreased due to excitotoxicity which may progress epileptogenesis as well (Putkonen et al., 2011). Metabotropic receptors may also have effects on epileptogenesis.

mGluRs may contribute to epileptogenesis; in a disease state group I mGluRs may be epileptogenic in nature when bound by glutamate whereas group II mGluRs promote antiepileptogenic effects when bound by glutamate (Sayin & Rutecki, 2003; Meldrum et al., 1999). Other processes may work to promote epileptogenesis such as a decrease in GABAergic interneurons (which could cause an increase in glutamate neurotransmission) and a decrease in membrane expressed GABA_B receptors (which could also cause an increase in glutamate neurotransmission) (Najm et al., 2001).

Several morphological changes in the hippocampus occur during epileptogenesis that are likely associated with glutamate dysregulation (Magloczky et al., 2000). Hippocampal sclerosis, shrinkage, and reactive gliosis have all been documented in epilepsy (Moore et al., 1999). Neuronal loss in hilar mossy cells, interneurons, and pyramidal neurons of the CA3 and CA1 are also observed in the granule cell layer (Magloczky et al., 2000; Proper et al., 2000). Evidence also exists suggesting that new gap junctions may form between small neuronal ensembles which may explain some of the synchronous firing in neurons during seizures (Bragin et al., 1999).

Aberrant rewiring in pyramidal cells of the CA1 and, from the granule cell layer to the CA3, also occurs (Magloczky et al., 2000; Proper et al., 2000). GABAergic sprouting in the DG and CA1 may occur as well; however, the pyramidal cell sprouting in the CA1 predominates and causes a feed-forward excitatory loop (Esclapez et al., 1999; Morin et al., 1999). Mossy fiber sprouting onto granule cells, interneuron dendrites in the supragranule layer, and to granule cells in the hilus also occurs and is speculated to create recurrent excitatory circuits (Ribak et al., 2000).

Sprouting is often thought to occur due to the loss of normal neuronal targets (Dalby & Mody, 2001). However, evidence suggests that sprouting may not be a cause of epileptogenesis but may be a result of it; thus, this should be taken in to consideration when interpreting these results (Gombos et al., 1999; Longo & Mello, 1998).

A lot of the changes seen in epileptogenesis are model specific; for example neuronal loss and synaptic restructuring are more prevalent in status epilepticus models as opposed to kindling models (Tuunanen & Pitkänen, 2000). Thus, differences between models should be taken in to account when attempting to uncover the changes that are universal to the process of epileptogenesis. Other limbic structures may also be damaged and contribute to epileptogenesis (see Bertram, 2009); however, only hippocampal data are presented here as it is the focus of this thesis.

Thesis Outline

This thesis is concerned with the effect neuronal presynaptic proteins may have on glutamate release and what affect that may have on the hippocampal trisynaptic circuit and the process of epileptogenesis. Chapter two discusses the materials, methods, instrumentation, methodology, and statistical analyses used in this thesis. Chapter three discusses the use of enzyme-based microelectrode arrays (MEAs) to measure glutamate in the DG, CA3, and CA1 in mice lacking a negative regulator of glutamate release, tomosyn. Glutamate was measured in hippocampal subregions in Tom^{+/+}, Tom^{+/-}, and Tom^{-/-} mice. The most robust finding was in the DG where an increase in spontaneous glutamate release, KCl-evoked glutamate peak amplitude, and KCl-evoked glutamate release were seen as tomosyn protein expression was decreased across genotype. Thus, as tomosyn expression decreases in the DG glutamate release increases; this may be why tomosyn mice have a kindling phenotype.

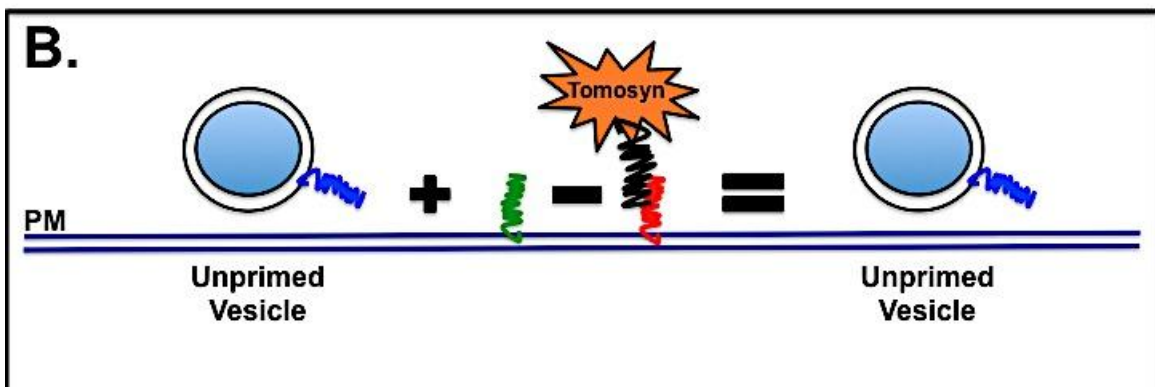
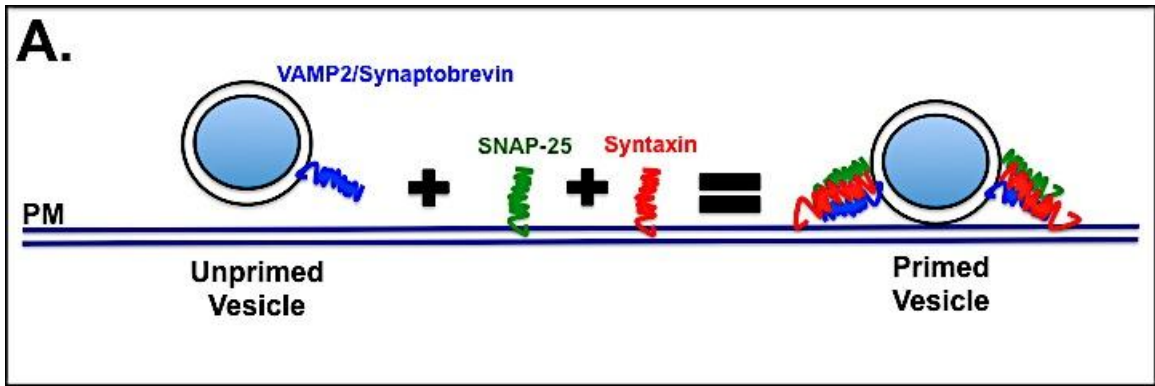


Figure 1.1 The Role of Tomosyn in Vesicle Priming

A. When tomosyn is not present, the vesicle membrane SNARE protein VAMP2/synaptobrevin can combine with the plasma membrane SNAREs, SNAP-25 and syntaxin, to create the 7S SNARE Complex and to prime the vesicle for release into the synapse. **B.** When tomosyn is present, it sequesters syntaxin and thus VAMP2/synaptobrevin, SNAP-25, and syntaxin cannot combine to form a primed vesicle for release into the synapse. Figure was adapted from Ashery et al. (2009).

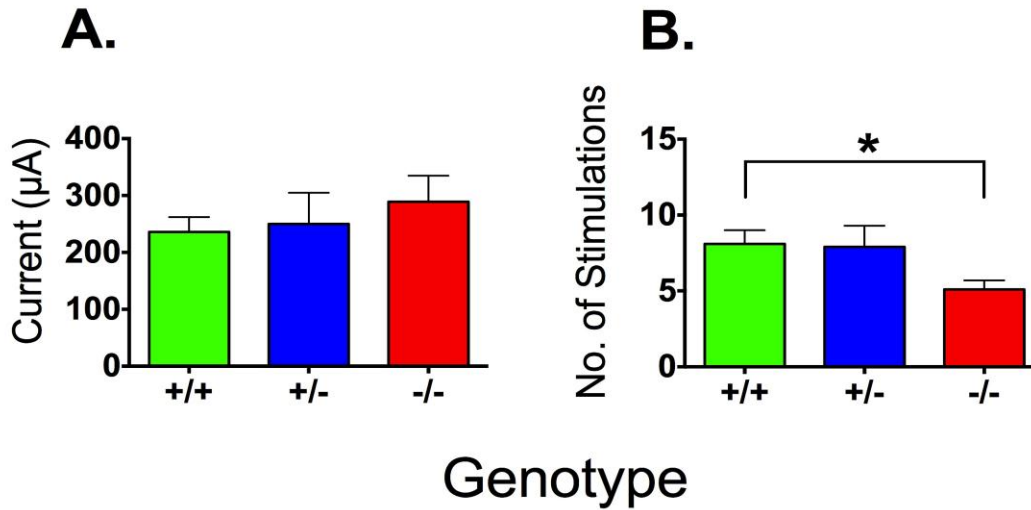


Figure 1.2 Current & Number of Stimulations Needed to Kindle/Genotype

A. The current needed to fully kindle Tom^{+/+} (n = 7), Tom^{+/-} (n = 7), and Tom^{-/-} (n = 14) animals was not significantly different between genotypes (one-way ANOVA; $F(2,25) = 0.37$, $p = 0.70$). **B.** Significantly fewer stimulations were required to kindle Tom^{-/-} (n = 14) mice compared to Tom^{+/+} (n = 7) mice (one-way ANOVA; $F(2,25) = 4.4$, $p = 0.024$; Student's t-test post-hoc, $p = 0.012$).

**Data courtesy of Dr. John T. Slevin, Dr. Sidney W. Whiteheart, and Ramona Alcala*

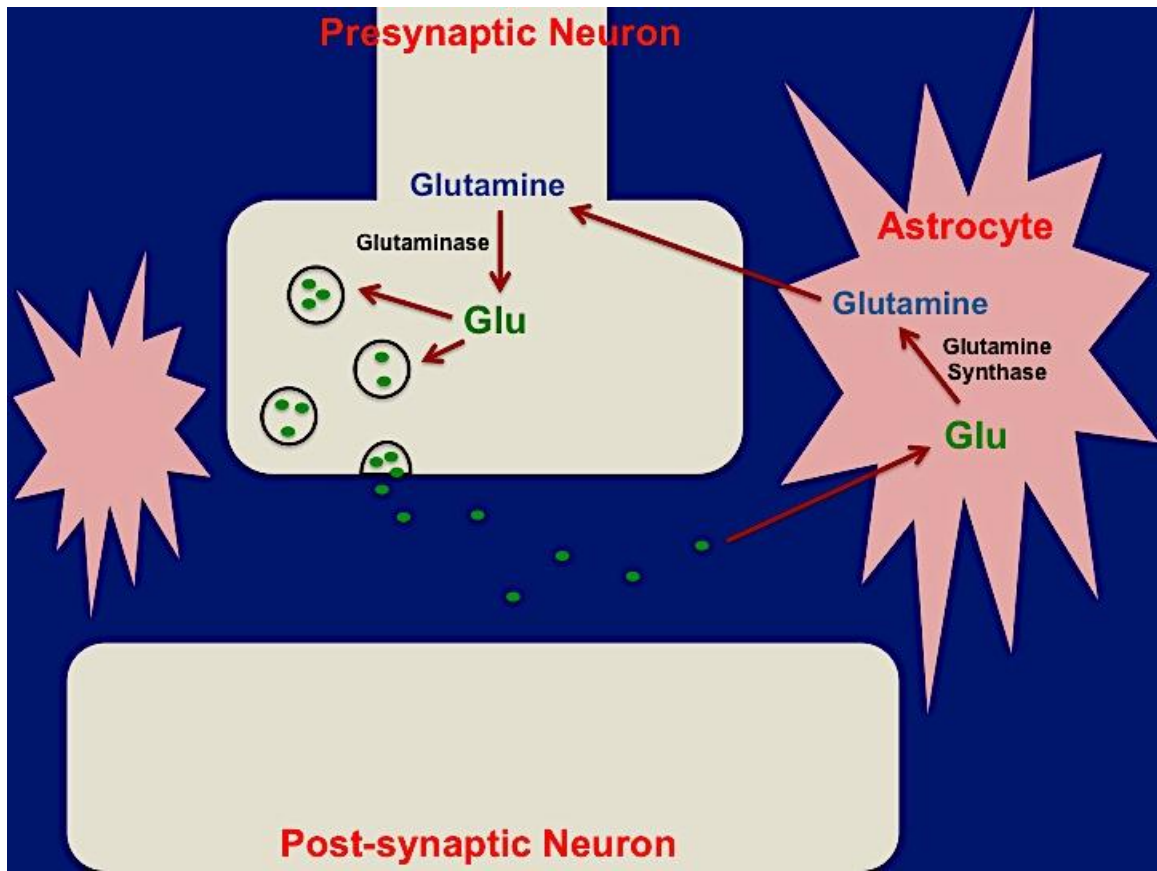


Figure 1.3 Glutamate Synthesis & Storage

Neurons take up glutamine in a Na^+ dependent fashion. Glutaminase then synthesizes glutamate from glutamine. Glutamate is then packaged in synaptic vesicles. Once glutamate is released it is taken up by astrocytes in a Na^+ dependent manner and converted to glutamine via glutamine synthase. Glutamine is then shuttled back to neurons and resynthesized to glutamate.

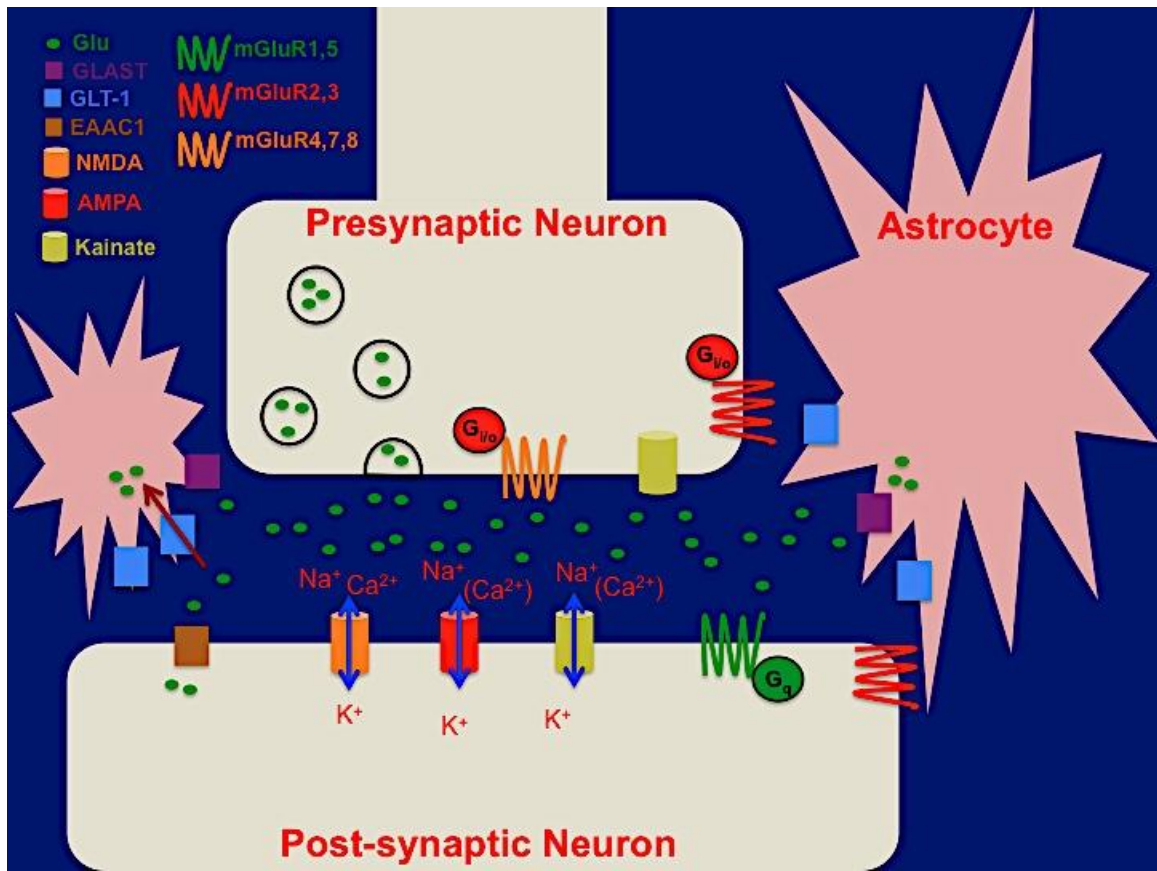


Figure 1.4 Typical Glutamate Synapse

Once glutamate is released into the synapse, it is free to bind to pre and post-synaptic receptors or to be removed by high affinity transporters on glial cells. Glutamate will cause cellular excitation when bound to NMDA, AMPA, Kainate or mGluR1,5 receptors. Glutamate will cause cellular inhibition when bound to mGluR2,3,4,7,8 receptors. Glutamate binding to inhibitory presynaptic mGluRs is an important negative feedback system for glutamate release/regulation.

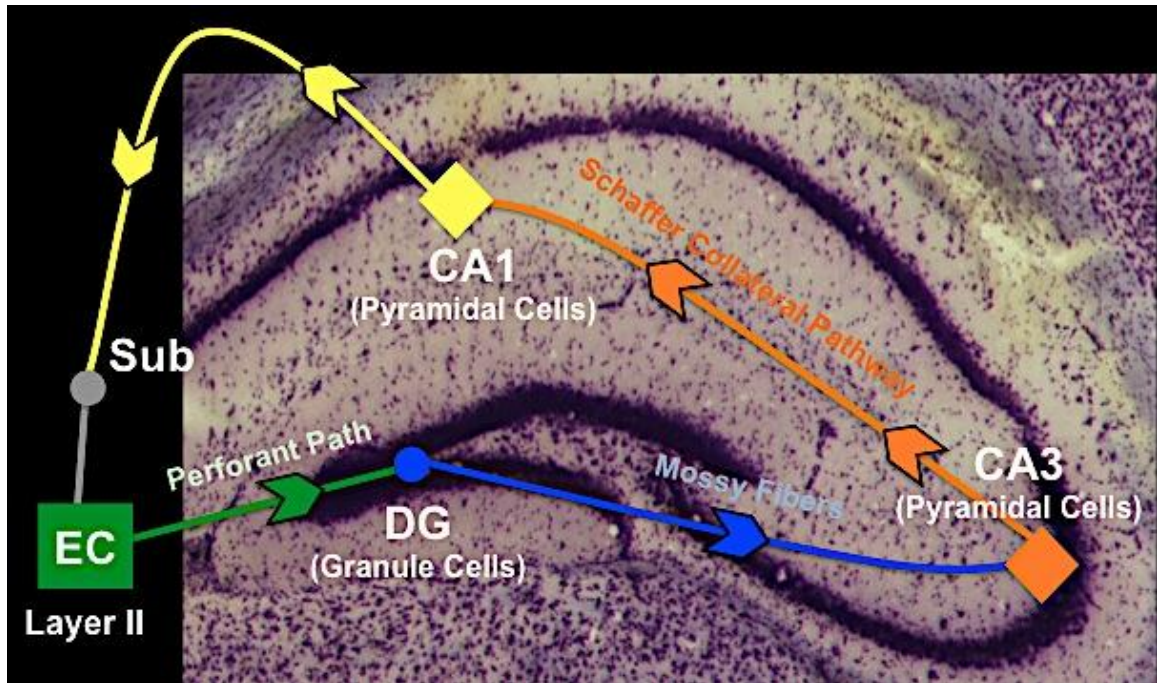


Figure 1.5 The Trisynaptic Loop

The trisynaptic loop is the unidirectional flow of glutamatergic neurotransmission in the hippocampus. The loop starts with the perforant path beginning in the entorhinal cortex (EC) and synapsing on the granule cells of the dentate gyrus (DG). Mossy axons then travel from the DG to synapse on the pyramidal cells in CA3. Signals are then sent through neurons of the bilateral Schaffer collaterals to synapse on pyramidal cells of the CA1 region of both hippocampi. The signals are then transmitted from the CA1 to the subiculum. From the subiculum signals return to the EC to complete the loop.

Chapter Two: Materials & Methods

Animals

Tomosyn Mice

Tomosyn mice were provided by Dr. Sidney Whiteheart (University of Kentucky). Experiments were performed on adult (20-37 g) Tom^{+/+} (n = 8), Tom^{+/-} (n = 8), and Tom^{-/-} (n = 9) mice 10-12 weeks of age. The genotype of animals used was determined by PCR analysis using DNA from tail clip biopsies. All mice were bred, maintained, and used according to the University of Kentucky IACUC approved protocol. Animals were housed in a 12-hour alternating light/dark cycle with food and water available *ad libitum*. All electrochemical glutamate recordings were performed during the light phase on average between 11:00 am-6:00 pm.

Principles of Electrochemistry

All glutamate recordings were performed using microelectrode arrays (MEA) selective for glutamate. The measuring sites of the MEAs are fabricated from inert platinum metal that can oxidize or reduced molecules of interest when a potential is applied versus an Ag/AgCl reference electrode. A custom built potentiostat and custom made software allowed for multiple, simultaneous measurements from different recording sites on a single MEA. Amperometry recordings were performed by applying a constant, fixed potential to the reference electrode which allows molecules to be oxidized at the platinum surfaces on the MEA (Gerhardt & Burmeister, 2000). The small currents resulting from these molecular oxidations/reductions are then amplified thus allowing the currents to be recorded. Amperometry allows the measurement of Faradaic current (which is linear and directly proportional to the concentration of molecules being oxidized/reduced) while minimizing non-Faradaic currents (background signals) (Burmeister & Gerhardt, 2001). Thus, this technique allows

for highly sensitive electrochemical measurements (micromolar) in the neuronal extracellular space on a real-time, sub-second scale (millisecond) (Burmeister et al., 2000).

Several neuromolecules such as dopamine (DA), norepinephrine (NE), serotonin (5-HT), nitric oxide (NO^{*}) ascorbic acid (AA), 3,4-dihydroxyphenylacetic acid (DOPAC), hydrogen peroxide (H₂O₂), and many others can be measured because of their electroactive properties (Hascup et al., 2007; Burmeister et al., 2000; Gerhardt & Burmeister, 2000 Gratton et al., 1989). Some molecules that are not electroactive at a low enough potential such as lactate, glucose, and glutamate can still be detected by the use of their respective oxidase enzymes by generating the reporter molecule H₂O₂ (Hascup et al., 2007; Burmeister & Gerhardt, 2001). Exclusion layers can also be plated (m-phenylenediamine [mPD]; excludes by size) or baked (Nafion[®]; excludes by charge [negatively charged thus repels anions]) on to the platinum surfaces of the MEA thus shielding the MEA surface from molecules that can interfere with currents from molecules of interest (Hascup et al., 2007).

Microelectrode Fabrication

The fabrication of these MEAs has been previously described (Hascup et al., 2007; Burmeister et al., 2002; Burmeister et al., 2000). In this study W4-style MEAs were used which have four platinum recording sites each measuring 20 μm x 150 μm in area. The four sites are arranged on the ceramic surface such that one pair of sites is spaced 100 μm above the other pair with 30 μm between the sites in a pair (Figure 2.1 A). This MEA configuration was selected for two reasons: (1) the small size of the platinum sites allowed for discrete placement of the recording sites in subregions of the hippocampus, (2) the design allowed for two sites to be easily coated with glutamate oxidase (GluOx) for detection of glutamate and for two sites to easily be coated with protein matrix to serve as the sentinel sites (Hascup et al., 2007; Burmeister et al., 2000). For all calibrations and anesthetized animal recordings an electrochemical measuring system

(FAST-16mk III, Quanteon, LLC, Nicholasville, KY) was used to perform constant voltage amperometry (+0.7 V vs. Ag/AgCl reference). Due to the ability of this system to collect data from the MEA at a high rate (up to 1000 Hz) and due to the small size of the MEA (microns) this technology allows for high spatial and temporal resolution compared to other techniques such as microdialysis (Hascup et al., 2007)

Microelectrode Preparation for Glutamate Recordings

All MEAs were made selective for glutamate as previously reported (Hascup et al., 2007; Burmeister et al., 2002). Briefly, the pair of sites closest to the tip of the electrode were coated with less than 1 μ L of enzyme solution (1% bovine serum albumin; 0.125% glutaraldehyde; 1 % GluOx) using a Hamilton microsyringe (80100; Hamilton Co.). The pair of sites furthest from the tip of the electrode were coated with less than 1 μ L of BSA/glutaraldehyde solution that was lacking the GluOx enzyme. Coating the electrode in this way allowed for glutamate to be measured at the site pairs closest to the tip of the electrode while only background signal/noise was measured at the sites furthest from the tip of the electrode (known as the sentinel sites); tonic glutamate measurements can then be obtained by subtracting the sentinel sites from the glutamate measuring sites (see 'Basal Glutamate Measurements' for more details) (Figure 2.2).

After the MEAs were coated with GluOx, they were allowed to cure for at least 48 hours. After curing, the MEAs were plated with mPD (5 mM; Acros Organics, New Jersey, USA) for 20 minutes and then calibrated and used immediately or within a timespan of 24 hours-2 weeks. The mPD is used to create a size exclusion layer on the platinum recording sites of the MEA, making the MEAs selective for glutamate, allowing only small molecules such as H₂O₂ to pass while excluding larger molecules such as AA, DA, and DOPAC (Hascup et al., 2007).

***In Vitro* MEA Calibration**

Although MEA fabrication and enzyme coating procedures are designed to induce as little error as possible, inconsistencies in the platinum recording sites and in the efficacy of the enzyme on the MEA surface exist. Thus, in order to get an accurate measure of glutamate concentrations in the brain, each MEA is calibrated with known concentrations of glutamate in order to ensure accurate *in vivo* glutamate measurements.

The platinum recording sites of the MEA and a glass Ag/AgCl reference electrode were submerged in 40 mL of phosphate-buffered saline (0.05 M PBS; pH 7.4) encased in a water bath set at approximately 37°C. After the MEA equilibrated to the PBS solution, 500 μ L of 20 mM AA (termed the 'interferent') was added to the PBS (beaker concentration = 250 μ M) to check the adequacy of the exclusion layer and to obtain a selectivity measurement of glutamate versus AA for the MEA. Selectivity measures of 20:1 or higher are adequate and are considered to block 95% of undesired molecules (see Hascup et al., 2007).

After the interferent was added, three serial 40 μ L additions of 20 mM glutamate (termed the 'analyte') were added to the PBS (beaker concentration = 20, 40, and 60 μ M respectively) to generate a standard curve which equates current from the oxidation of the reporter molecule, H₂O₂, to changes in known analyte concentrations (Burmeister & Gerhardt, 2001). The slope for this standard curve is calculated in nA/ μ M and the linearity of the line is calculated as an R²; the slope is considered to be the sensitivity of the MEA for glutamate (see Hascup et al., 2007). From the baseline measure and the slope the limit of detection (LOD) is calculated as well; this is considered to be the lowest concentration of glutamate the MEA can detect (the signal to noise ratio). For W4 MEAs slopes of 3.0 pA/ μ M or higher, LODs of 1.0 μ M or lower, and linearities of R² > 0.99 are considered adequate.

After the serial additions of glutamate a single 40 μ L addition of 2 mM DA was added to the PBS solution (beaker concentration = 2 μ M) to test the electrodes for their selectivity against DA. Lastly, a 40 μ L addition of 8.8 mM

H₂O₂ was added to the PBS (beaker concentration = 8.8 μM) to test the responsiveness of all the platinum sites to peroxide (Figure 2.3).

***In Vivo* Glutamate Measurements**

Basal Glutamate Measurements

Basal, tonic, or resting glutamate levels are caused by regulated release and uptake of glutamate (Danbolt, 2001). This measurement is the most basic measurement that can be obtained by the MEA. Data for this measure was collected in all animals in this study.

As mentioned above, the W4 MEA has four sites: two coated with GluOx to measure glutamate (measuring sites) and two coated with protein matrix that cannot measure glutamate (sentinel sites). This method allows us to subtract the background signal/noise caused by other electroactive substances and the charging current of the MEA surface (measured on all sites) away from the glutamate signal (measured only on the glutamate sensitive sites) thus leaving an accurate basal glutamate concentration (Figure 2.4); this method is termed self-referencing (Burmeister & Gerhardt, 2001; Day et al., 2006). By using this method we can isolate the current created by the reporter molecule generated from the enzymatic breakdown of glutamate. Basal glutamate measurements were calculated in micromolar concentrations by taking the current (nA) measured in the brain and dividing it by the MEA slope (nA/μM) obtained during the calibration (Quintero et al., 2007).

Evoked Glutamate Measurements

KCl-evoked glutamate measures were obtained by locally applying 100 nL of 70 mM KCl solution into distinct subregions of the hippocampus. KCl-evoked glutamate release can be thought of as the maximum amount of glutamate release possible from a subset of neurons per a given stimulation. Accurate KCl-evoked concentrations were obtained in μM concentrations through self-referencing (Figure 2.4).

Spontaneous Transient Glutamate Measurements

Because the MEA technology can record at a high sampling rate, rapid, spontaneous glutamate transients can be detected *in vivo* as described in Hascup et al. (2011a). These rapid events are usually small (below 1 μM in concentration), fast (1 second or less) naturally occurring glutamate bursts that are observed on the glutamate measuring sites but not on the sentinel sites (Hascup et al., 2011). The self-referencing technique was used to accurately measure glutamate transients in this study (Figure 2.5).

***In Vivo* Anesthetized Mice Recordings**

***In Vitro* MEA Calibration Specifications**

The W4 MEAs used in this study ($n = 25$) were calibrated according to the methods described above and had the following average parameters (mean \pm SD): slope (5.5 ± 2.1 pA/ μM); LOD (0.75 ± 0.39 μM); selectivity (508 ± 1057); R^2 (0.99). Note that these averages are roughly as good or better than published observations (Hascup et al., 2011b).

Surgical Procedures

Male Tom^{+/+}, Tom^{+/-}, and Tom^{-/-} mice were anesthetized using isoflurane (vaporizer 1-3%; flow 1 L/min) and placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA). A bilateral craniotomy was then performed by removing a square area of bone approximately 3 mm x 3 mm in size between bregma and lambda allowing access to the hippocampus. A small burr hole was then drilled out over the frontal cortex and an Ag/AgCl reference electrode was placed in the frontal cortex. The micropipette/MEA assembly was then attached to the stereotaxic frame and moved accordingly to target the DG, CA3, and CA1 hippocampal subregions (Figure 2.1 B).

Stereotaxic coordinates were adapted from Paxinos and Franklin (2008). Anterior-posterior and medial-lateral coordinates were taken from bregma, and dorsal-ventral coordinates were taken from the surface of the brain. The

following coordinates (in mm) were used: DG (AP: -2.3; ML: \pm 1.5; DV: -2.1); CA3 (AP: -2.3; ML: \pm 2.7; DV: -2.25); CA1 (AP: -2.3; ML: \pm 1.7; DV: -1.4).

Basal & Transient Glutamate Measurements

After the MEA equilibrated in the brain (approximately 20 minutes) basal and transient glutamate measures were collected for approximately 40 minutes in each brain area before any solutions were ejected. The order in which the subregions were targeted was counter-balanced to control for any order effects. Tonic and transient glutamate measures were collected in all animals. After 40 minutes of basal and transient glutamate measures, KCl was ejected into the brain regions and evoked glutamate measures were collected.

KCl-Evoked Glutamate Measurements

A single-barrel glass micropipette (1mm o.d.; 0.58 i.d.; A-M Systems Inc., Everett, WA) was pulled to an inner diameter of 10 μ m and was attached to the MEA using Sticky Wax (Kerr Lab Corporation, Orange, CA) (Figure 2.1 C). The pipette was positioned in the center of the four platinum sites 50 μ m-100 μ m from the surface of the MEA (Figure 2.1 D; Figure 2.1 E). The pipette placement allowed for the local application of 100 nL of 70 mM KCl (70 mM KCl, 79 mM NaCl, 2.5 CaCl₂; pH 7.4) in each subregion of the hippocampus to measure the maximum evoked glutamate release.

A Picospritzer III (Parker Hannifin Corp. NJ, USA) was attached to the micropipette via tubing and was used to precisely control the volume of KCl being locally ejected into each brain region. A dissecting microscope fitted with a calibrated reticule was used to measure the volume of KCl locally ejected into the brain (Friedemann & Gerhardt, 1992). In each subregion of the hippocampus 6-10 Glutamate peaks were evoked by approximately 100 nL of KCl per ejection. Evoked glutamate release was collected in all animals.

Confirmation of MEA Placement

At the end of every experiment, the micropipette waxed onto the MEA was filled with green ink (Special Green Ink; KOH-I-NOOR Co.) and the MEA was placed back into each of the hippocampal subregions where glutamate recordings took place. Careful consideration was taken to make sure the MEA

did not move on the stereotaxic frame while the pipette was being filled with ink. While putting the electrode back into the brain areas after the recordings had taken place was not ideal it was considered to be a good approximation of MEA placement especially considering that there was no other method to mark placement. Once the MEA was placed back into the brain the ink was ejected (approximately 500-600 nL) into each of the brain regions then the animal was euthanized and the brain was flash frozen using dry ice. Brains were cut into 40 μm thick slices using a cryostat and the slices were stained with Cresyl Violet (Sigma-Aldrich). Slices were then visually analyzed using a microscope (Carl Zeiss Co.) to confirm electrode placement in the DG, CA3, and CA1 (Figure 2.6). There were no clear indications that a brain region was missed thus no data were excluded due to incorrect electrode placement.

Data Analysis

Basal, transient, and KCl-evoked glutamate measures were recorded in current (nA) and divided by the slope from the calibration (nA/ μM) by the FAST Analysis MATLAB[®] software (MathWorks, Natick, MA.) to acquire the concentration of glutamate (μM) for each measure in each brain region (Quintero et al., 2007). Data were collected and analyzed for all measures in Tom^{+/+} (n = 7), Tom^{+/-} (n = 6), and Tom^{-/-} (n = 9) mice.

Tonic glutamate measures were analyzed using self-referencing before any KCl was ejected into the brain. Spontaneous transient measures were also analyzed during the 40-minute baseline before any KCl was ejected into the brain. Transient measures were analyzed by examining three specific measurements: (1) amplitude of the transient peak; (2) the area under the transient peak; (3) the number of transient peaks per a 10-minute bin.

KCl-evoked data was analyzed by examining the following specific measurements: (1) the amplitude of the evoked peak; (2) the area under the evoked peak (thought of as the total amount of glutamate released); (3) the time to rise of the evoked peak (T_{rise}) from baseline; (4) the first order rate of decay of the evoked peak (k^{-1} [sec^{-1}]; a measure of uptake) (Figure 2.4). The Fast Analysis software exported all data to a spreadsheet to be analyzed.

All data are presented as mean \pm SEM and was statistically analyzed using JMP[®]10 software (SAS Institute Inc., Cary, NC). One-way between subject ANOVAs and linear trend analyses were conducted for all glutamate measures. Outliers were excluded from analyses by using the Grubbs' test. One outlier was present for Tom^{+/+} animals, two outliers were present for Tom^{+/-} animals, and no outliers were present for Tom^{-/-} animals. The Tukey HSD post-hoc test was used to probe ANOVAs. Statistical significance was set at $p < 0.05$ for all statistical tests.

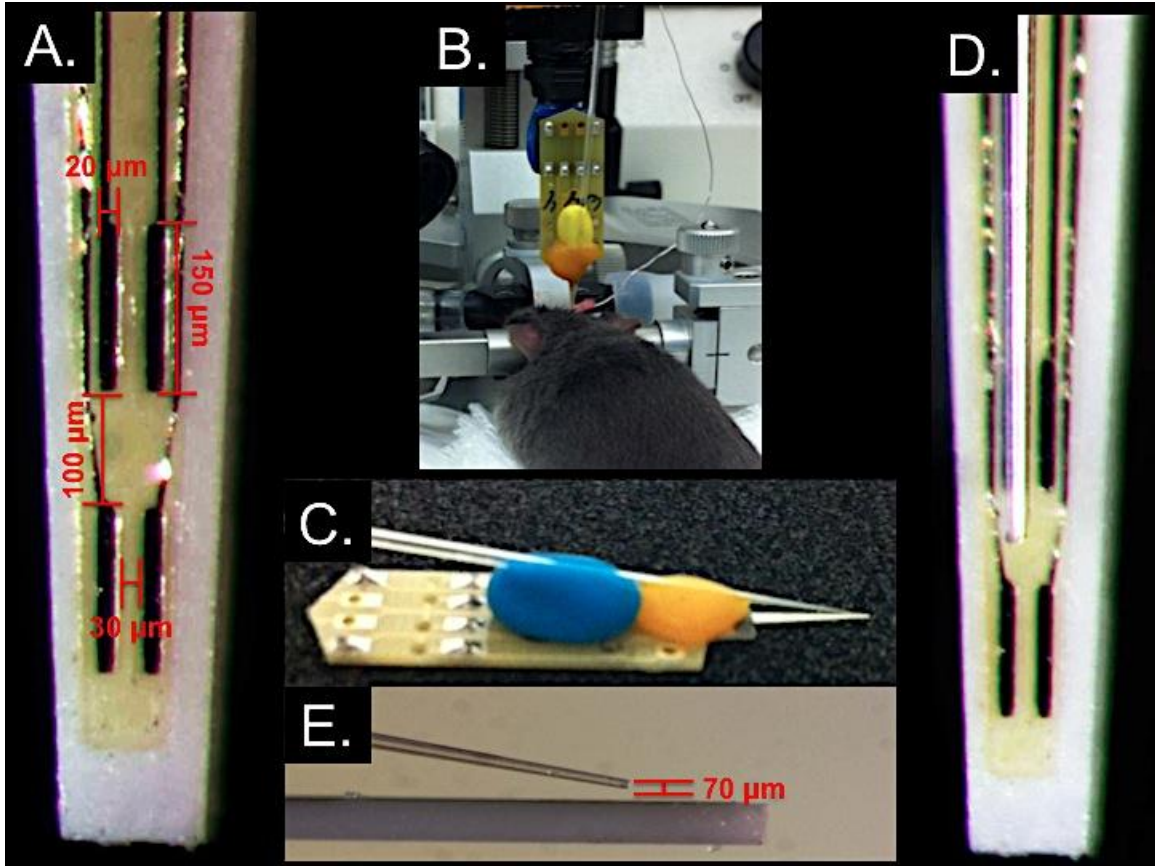


Figure 2.1 W4 MEA Setup

The W4 MEA is shown here to demonstrate the various ways it was used in this thesis. **A.** Close up of the W4 MEA shows the configuration of the platinum sites along with the measurements of the platinum sites ($20 \times 150 \mu\text{m}$), the distance the site pairs are from each other ($100 \mu\text{m}$), and the space between one member of a pair from the other ($30 \mu\text{m}$). **B.** W4 MEA implanted into the mouse hippocampus in the anesthetized setup. **C.** A micropipette waxed onto a W4 MEA using putty and Sticky Wax. **D.** A micropipette positioned in the middle of the four recording sites for local application of 70 mM KCl in the hippocampus. **E.** A micropipette positioned approximately $70 \mu\text{m}$ above the W4 MEA for local application of KCl into the hippocampus.

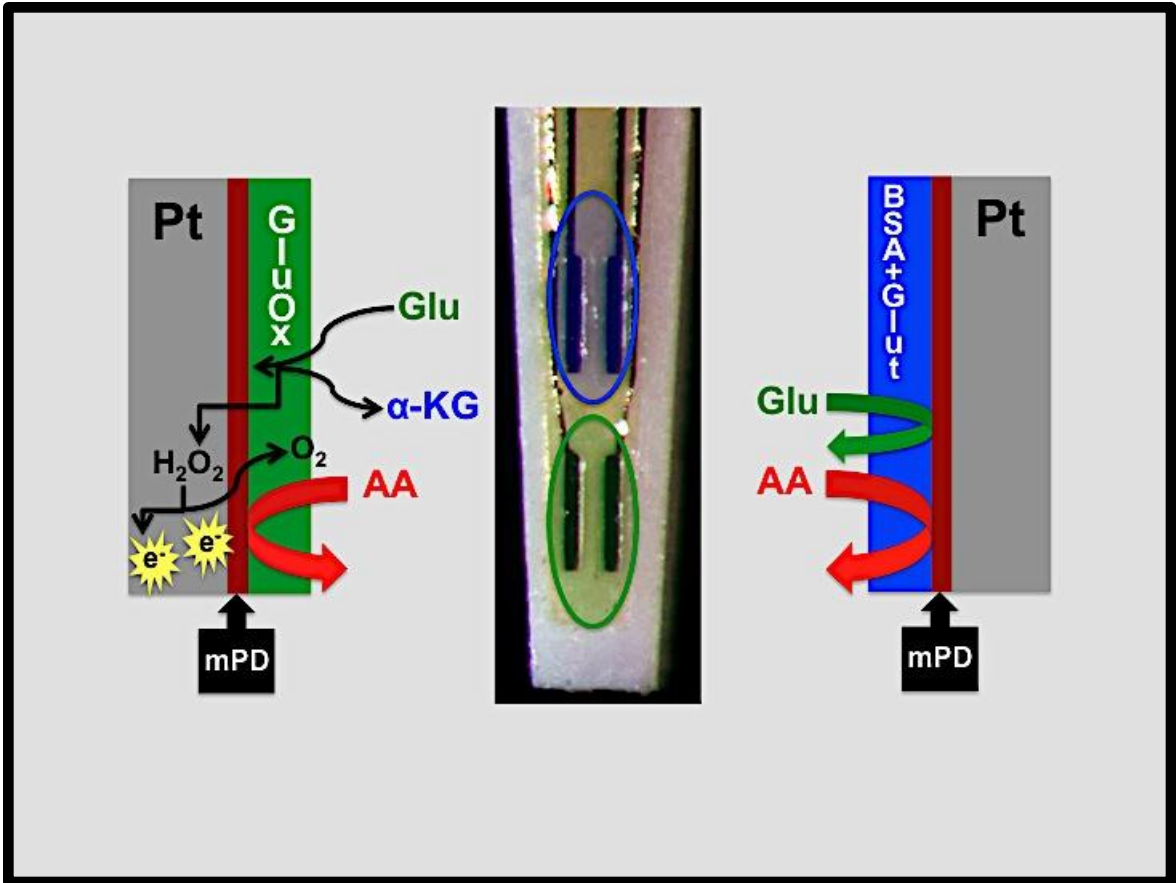


Figure 2.2 MEA Configuration for Self-Referenced Glutamate Measurements

W4 image showing m-phenylenediamine (mPD) exclusion layer, bovine serum albumin-glutaraldehyde protein matrix (BSA+Glut), and glutamate-oxidase (GluOx) active enzyme layer. **Green shaded sites:** contain GluOx and thus can create hydrogen peroxide (H_2O_2) from glutamate which can be oxidized by the MEA (receiving two electrons [e^-]). **Blue shaded sites:** sentinel sites that contain protein matrix and thus can only measure background current and not glutamate; the sentinel sites are subtracted from the glutamate recording sites to acquire accurate basal glutamate levels in the brain (termed self-referencing). mPD excludes ascorbic acid (AA) and other large molecules (DA; DOPAC) by size thus stopping them from reaching the platinum recording surfaces.

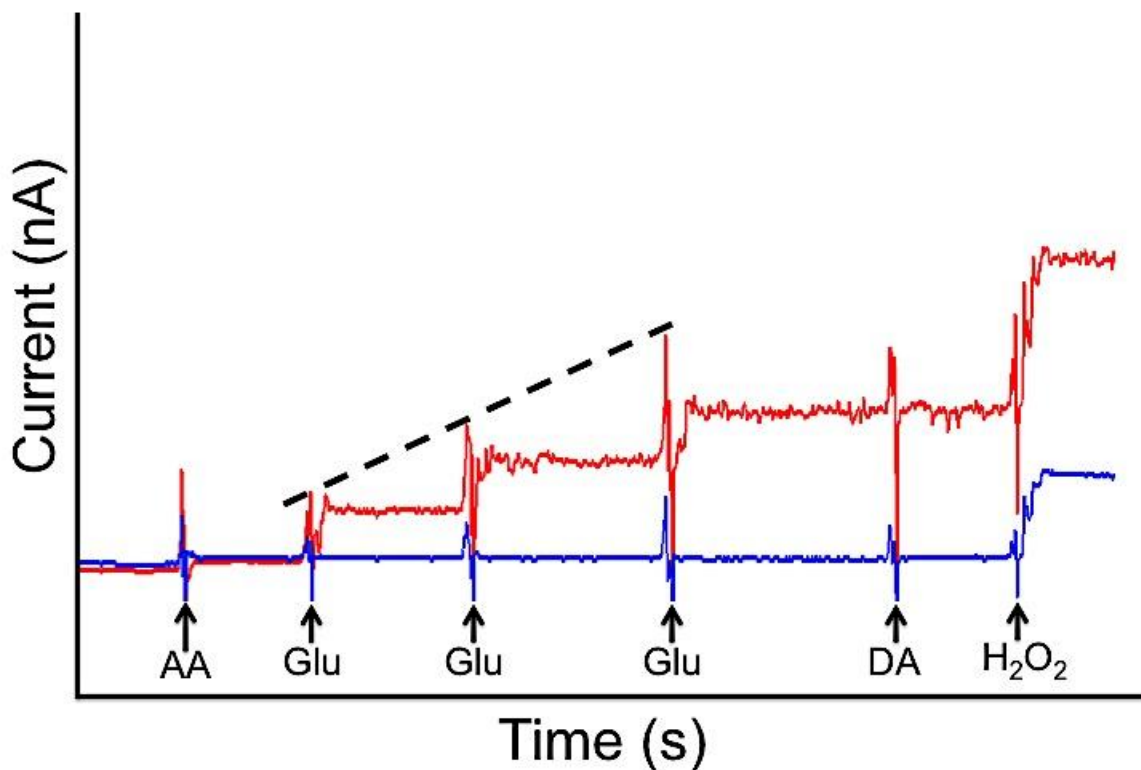


Figure 2.3 Glutamate Calibration

After the electrode equilibrated in the PBS solution an addition of ascorbic acid (AA; 250 μM) was added to the beaker to test the MEAs' selectivity against interferences. Three serial additions of glutamate (Glu) were then added to the beaker (20, 40, 60 μM respectively) to generate a standard curve (dashed line; $R^2 > 0.99$) from which a slope ($\text{nA}/\mu\text{M}$) can be generated. The slope represents the sensitivity of the MEA to glutamate and it can be used to equate changes in current (nA) to changes in concentration (μM) in the brain. Dopamine (DA; 2 μM) is added as a negative control. Hydrogen peroxide (H_2O_2 ; 8.8 μM) is added as a positive control.

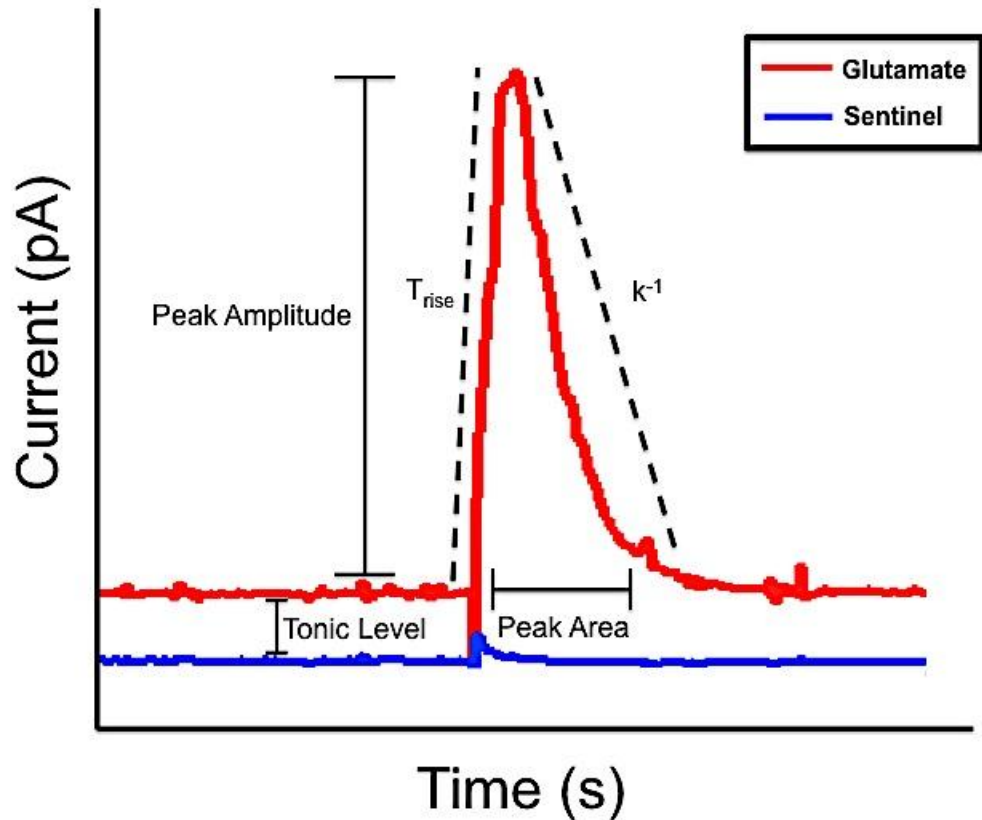


Figure 2.4 Glutamate Measurements

The various glutamate measures analyzed in this thesis. **Tonic Level:** the basal level of glutamate measured in the brain obtained by subtracting the sentinel sites from the glutamate recording sites. **Peak Amplitude:** the maximum amplitude of a KCl-evoked glutamate peak; an accurate concentration in the brain is calculated by self-referencing. **T_{rise} :** the time it takes a KCl-evoked glutamate peak to reach its maximum height in seconds. **Peak Area:** the area under a KCl-evoked glutamate peak in arbitrary units. **k^{-1} :** the glutamate uptake rate constant for a KCl-evoked peak in seconds⁻¹; the rate constant is calculated by fitting a first-order exponential curve to the decaying position of the evoked peak.

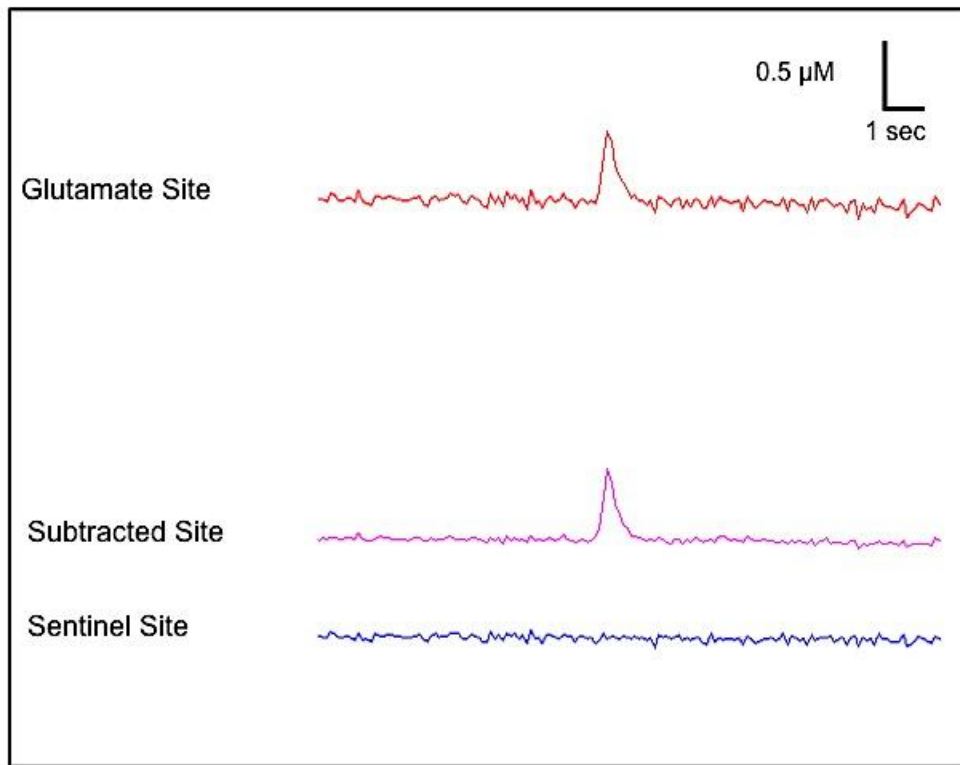


Figure 2.5 Spontaneous Glutamate Peak

An example of a naturally occurring spontaneous glutamate peak. Notice how the peak was observed on the glutamate site but not on the sentinel site. This is indicative of an actual glutamate peak as opposed to background current which would be present on both sites. The subtracted site shows the actual concentration (μM) of the spontaneous glutamate peak. Notice the small amplitude (approximately $0.5 \mu\text{M}$) and fast timespan (approximately 1 second) of the spontaneous peak.

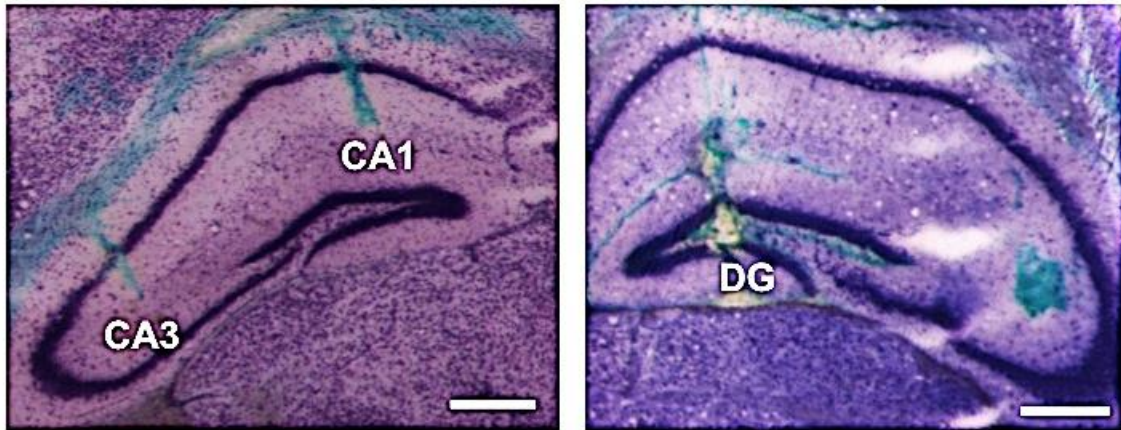


Figure 2.6 Histological Placement of MEA

A representative histological slice (40 μm) of the mouse hippocampus showing W4 MEA placement. **Left Panel:** green dye ejected via the micropipette waxed onto the W4 MEA shows electrode placement in the CA3 and CA1. **Right Panel:** green dye ejected via the micropipette waxed onto the W4 MEA shows electrode placement in the DG. Scale bar = 500 μm .

Chapter Three: Tomosyn Dysregulation Leads to Aberrant Glutamate Release in the Dentate Gyrus of the Hippocampus in a Murine Model of Epileptogenesis

Results

In this study we used electrochemical techniques with high spatial (μm) and temporal (4 Hz) resolution to explore *in vivo* glutamate neurotransmission in the DG, CA3, and CA1 subregions of the hippocampus in Tom^{+/+} (n = 7), Tom^{+/-} (n = 6), and Tom^{-/-} (n = 9) animals. Histological analysis confirmed MEA placements in the DG, CA3, and CA1 of all animals statistically analyzed. All animals were analyzed using one-way ANOVAs and linear trends. All data are presented as mean \pm SEM.

Tonic Glutamate Measurements

A loss of tomosyn does not affect tonic glutamate levels in the trisynaptic circuit. Tonic glutamate measurements were all approximately in the 1 to 3 μM range and showed no statistical difference between genotype (DG: $F(2,19) = 0.56$, $p = 0.58$; CA3: $F(2,19) = 0.70$, $p = 0.51$; CA1: $F(2,19) = 0.76$, $p = 0.48$) or within genotype (Tom^{+/+}: $F(2,18) = 0.11$, $p = 0.90$; Tom^{+/-}: $F(2,15) = 0.17$, $p = 0.84$; Tom^{-/-}: $F(2,24) = 0.029$, $p = 0.97$). No linear trends were present across genotype in the trisynaptic loop (DG: $t(19) = 0.75$, $p = 0.46$; CA3: $t(19) = 1.2$, $p = 0.25$; CA1: $t(19) = 1.2$, $p = 0.24$) (Figure 3.1). For a summary of averages and p -values see Table 3.1.

Spontaneous Peak Measures

Spontaneous glutamate recordings were collected during the 40 minute period before KCl ejections; the parameters analyzed were the amplitude of spontaneous peaks, the number of peaks per a 10-minute bin, and the area under spontaneous peaks. Tomosyn loss does not affect the amplitude of spontaneous glutamate peaks in the trisynaptic loop. The amplitude of the spontaneous peaks were approximately between 0.1 and 0.5 μM and showed no statistical difference between genotype (DG: $F(2,19) = 0.062$, $p = 0.94$; CA3:

$F(2,19) = 0.72, p = 0.50$; CA1: $F(2,19) = 1.3, p = 0.29$) or within genotype (Tom^{+/+}: $F(2,18) = 0.31, p = 0.74$; Tom^{+/-}: $F(2,15) = 0.55, p = 0.59$; Tom^{-/-}: $F(2,25) = 0.19, p = 0.83$). No linear trend was present across genotype (DG: $t(19) = 0.22, p = 0.83$; CA3: $t(19) = 1.1, p = 0.27$; CA1: $t(19) = 1.2, p = 0.23$) (Figure 3.2). See Table 3.2 for a summary of averages and p -values.

A roughly 50% knockdown of tomosyn appears to dysregulate glutamate bursting patterns between the CA3 and CA1 in Tom^{+/-} animals. The number of peaks per a 10-minute bin were approximately between 5 and 15 peaks with no statistical difference or linear trend in the hippocampus among genotype but with a statistical difference between the CA3 and CA1 in Tom^{+/-} animals (15 ± 2.6 vs. $5.4 \pm 1.8, F(2,19) = 5.0, p = 0.022$, Tukey post-hoc, $p = 0.029$) (Figure 3.3). For a summary of averages and p -values see Table 3.3.

Tomosyn loss causes more glutamate to be spontaneously released in a given release event as measured by the area under the glutamate peaks. The area under spontaneous peaks was approximately between 0.1 and 0.5 abu with no statistical difference between or within genotype in any of the brain regions; however, there was a linear trend in the DG across genotype (Tom^{+/+}: 0.13 ± 0.10 abu; Tom^{+/-}: 0.19 ± 0.11 abu; Tom^{-/-}: 0.33 ± 0.091 abu, $t(19) = 2.2, p = 0.040$) (Figure 3.4). See Table 3.4 for a summary of averages and p -values.

KCI-Evoked Glutamate Release

Tomosyn loss causes a higher concentration of maximum glutamate release during a given evoked event. The KCl-evoked peak amplitudes were approximately between 1 and 5 μ M with a statistical difference in the DG between Tom^{+/+} and Tom^{-/-} mice along with a linear trend across genotype in the DG (Tom^{+/+}: 1.7 ± 0.78 μ M; Tom^{+/-}: 2.3 ± 0.84 μ M; Tom^{-/-}: 4.2 ± 0.69 μ M, $F(2,19) = 4.0, p = 0.034$, Tukey post-hoc, $p = 0.029$; $t(19) = 2.8, p = 0.011$) (Figure 3.5). For a summary of averages and p -values see Table 3.5.

The amount of evoked glutamate released increases as tomosyn expression is decreased across genotype as measured by the area under evoked peaks. The area under KCl-evoked peaks was approximately between 5 and 26 abu with no statistical difference between or within genotype in any of the

brain regions in the hippocampus; however, there was a linear trend in the DG with an increase in total glutamate released as tomosyn was decreased across genotype (Tom^{+/+}: 8.1 ± 3.7 μM; Tom^{+/-}: 11 ± 4.0 μM; Tom^{-/-}: 19 ± 3.3, $t(19) = 2.0$, $p = 0.033$) (Figure 3.6). See Table 3.6 for a summary of averages and p -values.

A loss in tomosyn expression does not affect the time it takes for an evoked event to reach its maximum concentration; thus, tomosyn must not regulate the speed of glutamate release. The time to rise of KCl-evoked peaks was approximately between 2 and 3 seconds with no significant difference between genotype (DG: $F(2,19) = 0.27$, $p = 0.77$; CA3: $F(2,19) = 0.048$, $p = 0.95$; CA1: $F(2,19) = 0.41$, $p = 0.67$) or within genotype (Tom^{+/+}: $F(2,18) = 0.52$, $p = 0.60$; Tom^{+/-}: $F(2,15) = 0.12$, $p = 0.89$; Tom^{-/-}: $F(2,24) = 0.22$, $p = 0.81$). No linear trend was present across genotype in the trisynaptic loop (DG: $t(19) = 0.72$, $p = 0.48$; CA3: $t(19) = 0.070$, $p = 0.95$; CA1: $t(19) = -0.78$, $p = 0.45$) (Figure 3.7). See Table 3.7 for a summary of averages and p -values.

Tomosyn expression does not change the rate of decay of evoked glutamate peaks; thus, tomosyn has no effect on glutamate transport. The k^{-1} rate of decay of KCl-evoked peaks was approximately between 0.4 and 2 seconds⁻¹ with no significant differences between genotype (DG: $F(2,19) = 0.63$, $p = 0.54$; CA3: $F(2,19) = 0.81$, $p = 0.46$; CA1: $F(2,19) = 0.049$, $p = 0.95$) or within genotype (Tom^{+/+}: $F(2,18) = 0.43$, $p = 0.66$; Tom^{+/-}: $F(2,15) = 0.67$, $p = 0.52$; Tom^{-/-}: $F(2,24) = 0.22$, $p = 0.81$). No linear trend was present across genotype in any of the brain regions (DG: $t(19) = -1.1$, $p = 0.28$; CA3: $t(19) = -0.92$, $p = 0.37$; CA1: $t(19) = 0.30$, $p = 0.77$) (Figure 3.8). For a summary of averages and p -values see Table 3.8.

Discussion

In this study basal glutamate levels, the peak amplitude of spontaneous glutamate peaks, and the T_{rise} and k^{-1} of KCl-evoked glutamate peaks showed no significant difference or linear trend across or within Tom^{+/+}, Tom^{+/-}, or Tom^{-/-} mice. However, we observed a significant increase in the maximum

concentration of evoked peaks in Tom^{-/-} mice compared to Tom^{+/+} mice in the DG. We also observed significant differences between the number of spontaneous peaks per 10-minute bin between the CA3 and CA1 in Tom^{+/-} mice with the CA3 showing significantly more peaks than the CA1. Furthermore, positive linear trends in the DG for the area under spontaneous glutamate peaks, the amplitude of KCl-evoked peaks, and the area under KCl-evoked glutamate peaks were also detected (suggesting that as tomosyn expression was decreased across genotype more glutamate was released and a higher concentration was present in the synapse). Taken together these results suggest that a reduction in tomosyn expression leads to an increase in glutamate release, especially in the DG.

Tomosyn Does Not Alter Tonic Glutamate Levels

We observed no statistical difference or linear trend in tonic glutamate measurements in the hippocampus. This is consistent with what has been found using MEA technology to measure glutamate in kindled rats and in the VAMP2^{+/-} genetic mouse model (Matveeva et al., 2011a; Matveeva et al., 2011b). However, these findings are inconsistent with those using other techniques such as microdialysis in different epileptogenic models.

Using microdialysis, Ueda et al. (2001) found an increase in basal glutamate levels in the ventral hippocampus in a kainic acid model of epileptogenesis. The differences between these results and our study may be reconciled by the fact that MEAs have higher spatial (μm vs. mm) and temporal (ms vs min.) resolution thus what may look like basal changes over minutes may not actually be so when one looks at the changes on a timescale that is more accurate to neurotransmitter release; for example we had an increase in the amount of glutamate released in a given spontaneous peak. Microdialysis does not collect data fast enough to detect these spontaneous peaks. Thus, what may look like an increase in tonic glutamate levels using microdialysis may actually be an increase in the amount of glutamate released per a spontaneous peak. It is also possible that the changes seen in basal levels are model specific (Tuunanen

& Pitkänen, 2000), thus explaining why they are seen in the kainic acid model but not in the tomosyn genetic model or the kindling model.

Furthermore, evidence suggests that certain anesthesia's can have effects on resting glutamate levels (Rutherford et al., 2007). Considering Ueda et al. (2001) used sodium pentobarbital as an anesthetic and we used isoflurane it is possible that differences in basal levels were due to differences in the anesthetic used. Nevertheless, more work in unanesthetized freely moving mice in different models of epileptogenesis are needed to know for certain what effects, if any, different anesthetics may have on basal glutamate levels.

Tomosyn Does Alter Spontaneous Glutamate Release

No significant difference or linear trend was found in the amplitude of spontaneous peaks in the trisynaptic loop. This is a similar result to that presented in Matveeva et al. (2011a) where no significant difference was seen in the amplitude of spontaneous peaks in kindled rats compared to control rats in the hippocampus contralateral to where the kindling electrode was placed. However, that same study did find a significant difference in spontaneous amplitude measurements in the DG and CA3 between kindled and control animals ipsilateral to where the kindling electrode was placed. Thus, it is possible that spontaneous amplitude changes only exist in the hemisphere of the brain where the kindling stimulus occurs and that these changes are dependent more on kindling than tomosyn protein expression.

We found a significant difference in the number of peaks per 10-minute bin in Tom^{+/-} mice between the CA3 and CA1. In contrast, Matveeva et al. (2011a) found a difference in peaks per 5-minute bin between the DG and CA1 in the hippocampal hemisphere ipsilateral to where the kindling electrode was placed in kindled rats. However, no difference between the hippocampal hemisphere contralateral to the stimulating electrode in kindled rats or in either hemisphere of sham control rats was seen. Considering that our tomosyn mice resemble non-kindled controls, it is possible that a roughly 50% reduction in tomosyn is the reason for the difference in peaks per 10-minute bin. Further, it is also possible that if we were to analyze our data using 5-minute bins as opposed

to 10-minute bins these data may look similar; however, we chose to use 10-minute bins because we collected spontaneous data for a longer time period than Matveeva et al. (2011a) thus we did not think we needed to increase our peak per bin resolution further by using 5-minute bins.

A more interesting question is why Tom^{+/-} mice show dysregulation in this measure and Tom^{-/-} mice do not. This difference may be because several different proteins also regulate glutamate release (Matveeva et al., 2007). Thus, in Tom^{+/-} mice where there is roughly a 50% reduction in tomosyn, the compensatory response from other proteins may not be as robust as it is in Tom^{-/-} mice because some tomosyn is still present in heterozygous animals. Further, it is unlikely that these differences are due to differential expression of tomosyn in these brain regions (Barak et al., 2010) because the difference in 10-minute bins between the CA3 and CA1 is not seen in the wild-type tomosyn mice. Thus, some kind of compensatory response that occurred in Tom^{-/-} mice but not in Tom^{+/-} mice is likely the reason for the dysregulation seen in the heterozygotes.

A positive linear trend in the DG as tomosyn was decreased across genotype for the area measured under spontaneous peaks was also observed. The area under spontaneous peaks can be thought of as the total amount of glutamate released (Matveeva et al., 2011b). Thus, decreasing tomosyn expression increases the total amount of glutamate released by neurons in the DG while not changing the peak amplitude. It is possible that a compensatory response to a loss in tomosyn is greater in the CA3 and CA1 than in the DG explaining why an effect is seen in the DG but not the CA3 or CA1. Nevertheless, more experimentation is needed to explain why this difference may exist.

Tomosyn Alters KCI-Evoked Glutamate Release

A significant difference between Tom^{+/+} and Tom^{-/-} mice as well as a positive linear trend as tomosyn expression was decreased across genotype in KCI-evoked glutamate release in the DG was observed. Note that this finding is not isolated to our methodology or model as other groups have found similar

results using microdialysis in the hippocampus of rats with kainic acid and cocaine-kindled induced epileptogenesis (Kaminski et al., 2011; Ueda et al., 2001). Further, using MEAs to measure glutamate in the hippocampus of VAMP2^{+/-} mice with a kindling resistant phenotype, Matveeva et al. (2011b) found a decrease in the amplitude of KCI-evoked glutamate peaks in the DG and CA3. Considering that the tomosyn kindling phenotype is opposite the VAMP2^{+/-} model, it is not surprising to see an increase in the amplitude of KCI-evoked glutamate peaks at least in the DG. Considering that changes in the hippocampal glutamate system occur during kindling (see Matveeva et al., 2011a) differences are likely seen in this measure simply because the KCI ejections challenge an already vulnerable system.

We also observed a positive linear trend in the total amount of glutamate released (as measured by the area under evoked peaks) in the DG as tomosyn expression was decreased across genotype. In VAMP2^{+/-} mice a decrease in evoked area was seen in the CA3; it not surprising that the reverse is seen in tomosyn mice, albeit the effect is seen in the DG instead of the CA3 (Matveeva et al., 2011). We did not observe changes in the time to rise or k^{-1} decay rate of the KCI-evoked peaks. Thus, it seems that the increase in peak area is due to an increase in quantal glutamate release and not a difference in the time needed to reach maximum release amplitude or in uptake.

These changes in phasic glutamate release may be seen exclusively in the DG rather than CA3 and CA1 due to the role of the DG as 'the gatekeeper' of the trisynaptic loop (Tammaing et al., 2012). Thus, it is possible that while dysregulation happens in the DG, other compensatory responses may take place further downstream in the CA3 and CA1 that attenuate this glutamate dysregulation; a similar trend has been seen in the trisynaptic circuit with the amplitude of spontaneous glutamate peaks in kindled rats (Matveeva et al., 2011a). However, more experiments will need to be conducted to know exactly what those differential compensatory responses may be.

Conclusions

This study suggests that tomosyn has a role as a negative regulator of glutamate release, particularly in the DG of the hippocampus. Taken together with the fact that tomosyn knockout animals show a kindling phenotype, it suggests that a dysfunction in this protein may promote the formation and propagation of epilepsy. In addition to the differences found, it is possible that other differences may exist that could be elucidated by changing some of the experimental procedures.

It is possible that because our animals were tomosyn deficient from birth compensatory responses occurred, at least in the CA3 and CA1, causing no effects in these brain regions in the knockout mice. Thus, perhaps conditionally knocking out tomosyn expression in adult mice may make it easier to elucidate the range of effects this protein can have on glutamate neurotransmission. This ideas could be incorporated into future experiments to potentially improve our studies.

In the future, we plan to measure glutamate in kindled tomosyn animals to assess what glutamatergic changes may have occurred in these animals after kindling. We also plan to use the anti-seizure medication levetiracetam, which may affect various presynaptic proteins associated with glutamate release, in tomosyn and other genetic mice models in attempt to reverse the kindling phenotype completely or at the least attenuate the kindling process. We would also like to perform experiments on adult mice whose proteins are conditionally knocked out (allowing us to perhaps see a broader range of possible effects these proteins may have on the glutamate system). In these ways, we would be able to further elucidate the mechanisms of epileptogenesis and to explore potential ways to attenuate or eradicate the process.

In the United States alone, epilepsy affects millions of adults and thousands of children every year. To date there is no cure for epilepsy and the current treatments have varying degrees of efficacy. The processes the brain undergoes to develop epilepsy are unknown. However, by studying

epileptogenesis, it is possible that we may uncover these mechanisms and find treatments to abort the process. To this end, this thesis provides electrochemical data of aberrant hippocampal glutamate activity in an epileptic mouse phenotype that may provide clues from which we and others can design further experiments to answer the question "whereby epileptogenesis?".

Table 3.1 Tonic Glutamate in Tomosyn Mice (μM)

Brain Region	Tom^{+/+}	Tom^{+/-}	Tom^{-/-}	One-way ANOVA	Linear Trend
DG	1.8 \pm 0.43	1.7 \pm 0.47	2.2 \pm 0.38	$p = 0.58$	$p = 0.46$
CA3	1.5 \pm 0.40	1.8 \pm 0.43	2.4 \pm 0.35	$p = 0.51$	$p = 0.25$
CA1	1.5 \pm 0.62	2.0 \pm 0.67	2.6 \pm 0.55	$p = 0.48$	$p = 0.24$
One-way ANOVA	$p = 0.90$	$p = 0.84$	$p = 0.97$		

All data presented as mean \pm SEM

Table 3.2 Amplitude of Spontaneous Glutamate Peaks in Tomosyn Mice (μM)

Brain Region	Tom^{+/+}	Tom^{+/-}	Tom^{-/-}	One-way ANOVA	Linear Trend
DG	0.23 \pm 0.065	0.26 \pm 0.070	0.26 \pm 0.058	$p = 0.94$	$p = 0.83$
CA3	0.19 \pm 0.066	0.26 \pm 0.072	0.31 \pm 0.058	$p = 0.50$	$p = 0.27$
CA1	0.24 \pm 0.18	0.49 \pm 0.20	0.47 \pm 0.16	$p = 0.29$	$p = 0.23$
One-way ANOVA	$p = 0.74$	$p = 0.59$	$p = 0.83$		

All data presented as mean \pm SEM

Table 3.3 Spontaneous Glutamate Peaks/10-Minute bin in Tomosyn Mice

Brain Region	Tom^{+/+}	Tom^{+/-}	Tom^{-/-}	One-way ANOVA	Linear Trend
DG	12 ± 2.1	13 ± 2.3	9.8 ± 1.8	<i>p</i> = 0.51	<i>p</i> = 0.44
CA3	12 ± 2.4	15 ± 2.6	14 ± 2.1	<i>p</i> = 0.78	<i>p</i> = 0.58
CA1	9.9 ± 1.7	5.4 ± 1.8	8.2 ± 1.5	<i>p</i> = 0.20	<i>p</i> = 0.60
One-way ANOVA	<i>p</i> = 0.70	* <i>p</i> = 0.022	<i>p</i> = 0.066		

All data presented as mean ± SEM
 *Significance

Table 3.4 Area Under Spontaneous Glutamate Peaks in Tomosyn Mice (abu)

Brain Region	Tom ^{+/+}	Tom ^{+/-}	Tom ^{-/-}	One-way ANOVA	Linear Trend
DG	0.13 ± 0.10	0.19 ± 0.11	0.33 ± 0.091	<i>p</i> = 0.11	* <i>p</i> = 0.040
CA3	0.42 ± 0.18	0.18 ± 0.19	0.21 ± 0.15	<i>p</i> = 0.87	<i>p</i> = 0.84
CA1	0.30 ± 0.19	0.43 ± 0.20	0.39 ± 0.17	<i>p</i> = 0.35	<i>p</i> = 0.46
One-way ANOVA	<i>p</i> = 0.59	<i>p</i> = 0.16	<i>p</i> = 0.90		

All data presented as mean ± SEM

*Linear Trend

Table 3.5 Amplitude of KCl-Evoked Peaks in Tomosyn Mice (μM)

Brain Region	Tom^{+/+}	Tom^{+/-}	Tom^{-/-}	One-way ANOVA	Linear Trend
DG	1.7 \pm 0.78	2.3 \pm 0.84	4.2 \pm 0.69	* $p = 0.034$	* $p = 0.011$
CA3	2.1 \pm 0.42	2.8 \pm 0.45	2.3 \pm 0.37	$p = 0.40$	$p = 0.75$
CA1	3.4 \pm 1.1	3.6 \pm 1.2	2.5 \pm 0.97	$p = 0.81$	$p = 0.83$
One-way ANOVA	$p = 0.75$	$p = 0.79$	$p = 0.18$		

All data presented as mean \pm SEM

*Significance & Linear Trend

Table 3.6 Area Under KCI-Evoked Peaks in Tomosyn Mice (abu)

Brain Region	Tom^{+/+}	Tom^{+/-}	Tom^{-/-}	One-way ANOVA	Linear Trend
DG	8.1 ± 3.7	11 ± 4.0	19 ± 3.3	<i>p</i> = 0.095	* <i>p</i> = 0.033
CA3	5.8 ± 2.8	9.8 ± 3.0	11 ± 2.5	<i>p</i> = 0.25	<i>p</i> = 0.21
CA1	26 ± 7.5	9.4 ± 8.1	8.4 ± 6.6	<i>p</i> = 0.55	<i>p</i> = 0.28
One-way ANOVA	<i>p</i> = 0.26	<i>p</i> = 0.78	<i>p</i> = 0.095		

All data presented as mean ± SEM

*Linear Trend

Table 3.7 Time to Rise of KCl-Evoked Peaks in Tomosyn Mice (sec)

Brain Region	Tom^{+/+}	Tom^{+/-}	Tom^{-/-}	One-way ANOVA	Linear Trend
DG	2.2 ± 0.61	2.5 ± 0.66	2.9 ± 0.54	<i>p</i> = 0.77	<i>p</i> = 0.48
CA3	2.6 ± 0.63	2.4 ± 0.69	2.9 ± 0.56	<i>p</i> = 0.95	<i>p</i> = 0.95
CA1	2.7 ± 0.51	2.3 ± 0.55	2.4 ± 0.45	<i>p</i> = 0.67	<i>p</i> = 0.45
One-way ANOVA	<i>p</i> = 0.60	<i>p</i> = 0.89	<i>p</i> = 0.81		

All data presented as mean ± SEM

Table 3.8 Decay Rate of KCl-Evoked Peaks in Tomosyn Mice (sec⁻¹)

Brain Region	Tom^{+/+}	Tom^{+/-}	Tom^{-/-}	One-way ANOVA	Linear Trend
DG	1.7 ± 0.62	0.86 ± 0.67	0.44 ± 0.55	<i>p</i> = 0.54	<i>p</i> = 0.28
CA3	0.89 ± 0.43	1.2 ± 0.46	0.89 ± 0.38	<i>p</i> = 0.46	<i>p</i> = 0.37
CA1	0.58 ± 0.27	0.59 ± 0.30	0.79 ± 0.24	<i>p</i> = 0.95	<i>p</i> = 0.77
One-way ANOVA	<i>p</i> = 0.66	<i>p</i> = 0.52	<i>p</i> = 0.81		

All data presented as mean ± SEM

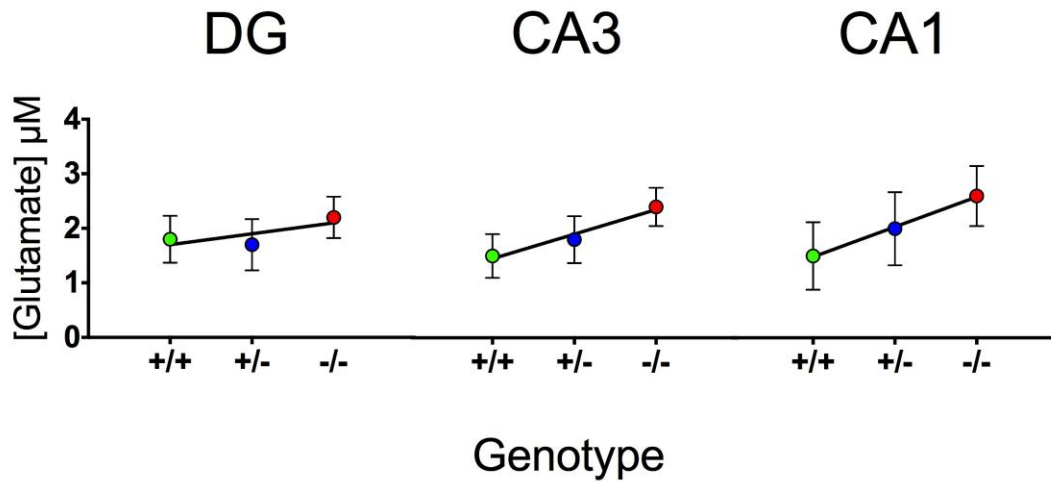


Figure 3.1 Tonic Glutamate is Not Different in Tomosyn^{-/-} Mice

No significant difference was observed in tonic glutamate levels among or within genotype. There was no linear trend in tonic glutamate levels across genotype. These data suggest that a loss of tomosyn does not affect glutamate levels in the trisynaptic circuit.

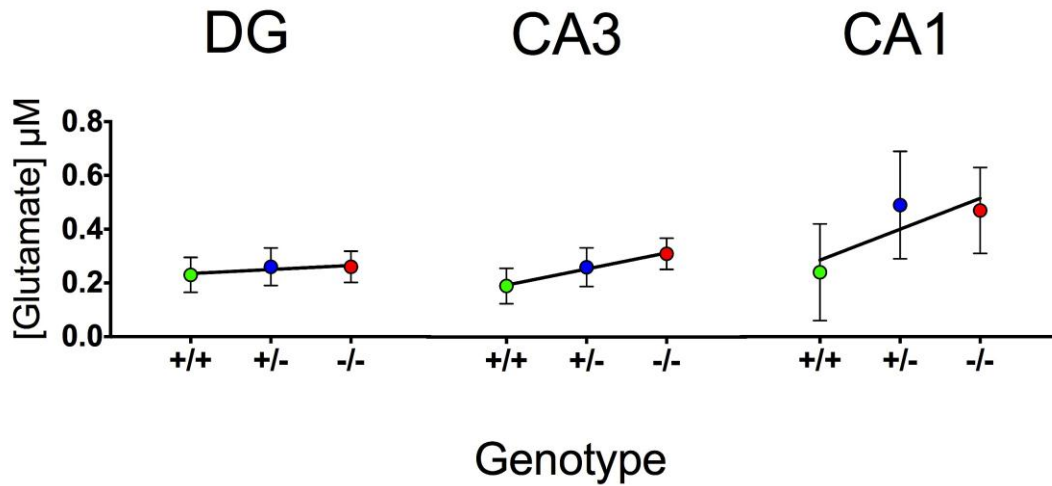


Figure 3.2 Amplitude of Spontaneous Glutamate Peaks is No Different in Tomosyn^{-/-} Mice

No significant differences were seen in the amplitude of spontaneous glutamate peaks between or within genotype. No linear trend was seen in tonic glutamate levels across genotype. Taken together, tomosyn does not seem to affect the amplitude of spontaneous glutamate peaks in the trisynaptic circuit.

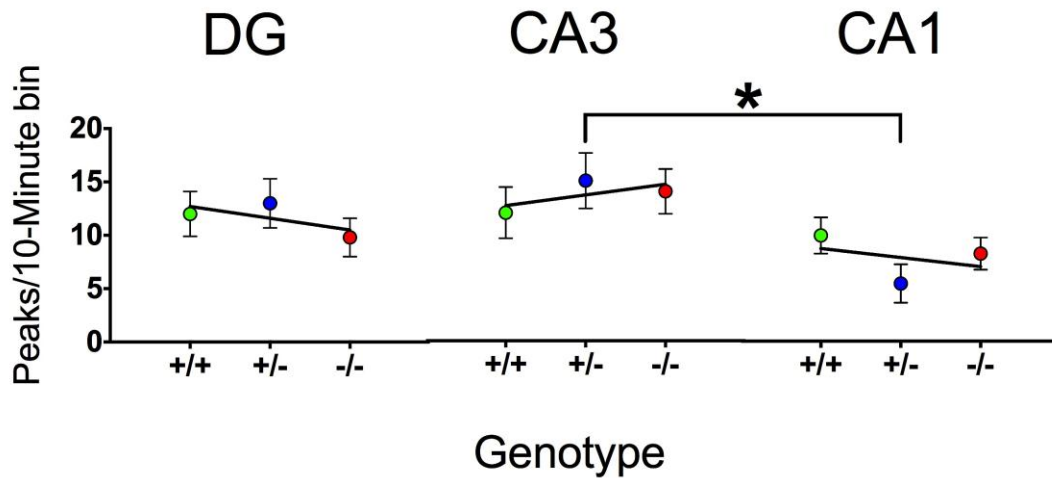


Figure 3.3 The Number of Spontaneous Peaks/10-Minute bin is Different Between the CA3 & CA1 in Tomosyn^{+/-} Mice

No Significant difference or linear trend was seen between genotype in the trisynaptic circuit. A significant difference was observed in Tom^{+/-} animals between the CA3 and CA1 suggesting that approximately a 50% knockdown in tomosyn causes a dysregulation in glutamate burst firing patterns in the CA3 and CA1. Specifically, this 50% decrease in tomosyn seemed to increase the firing pattern in the CA3 and decrease it in the CA1.

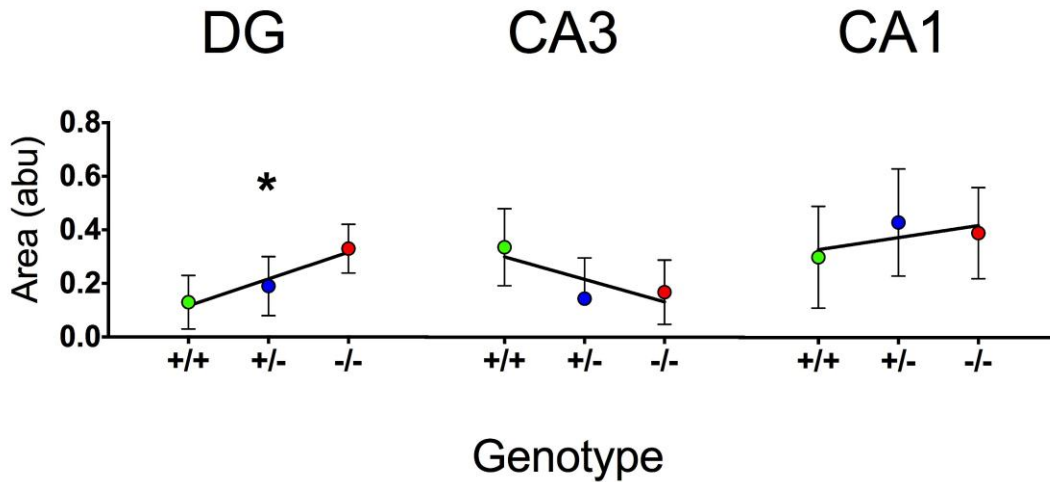


Figure 3.4 The Total Amount of Glutamate Released in Each Spontaneous Peak is Increased as Tomosyn is Decreased Across Genotype in the DG

No significant difference was observed in the trisynaptic circuit between genotype in the total amount of glutamate released in a spontaneous peak. A linear trend was observed in the DG across genotype for this measure suggesting that as tomosyn decreases more glutamate is released in each spontaneous peak in the DG.

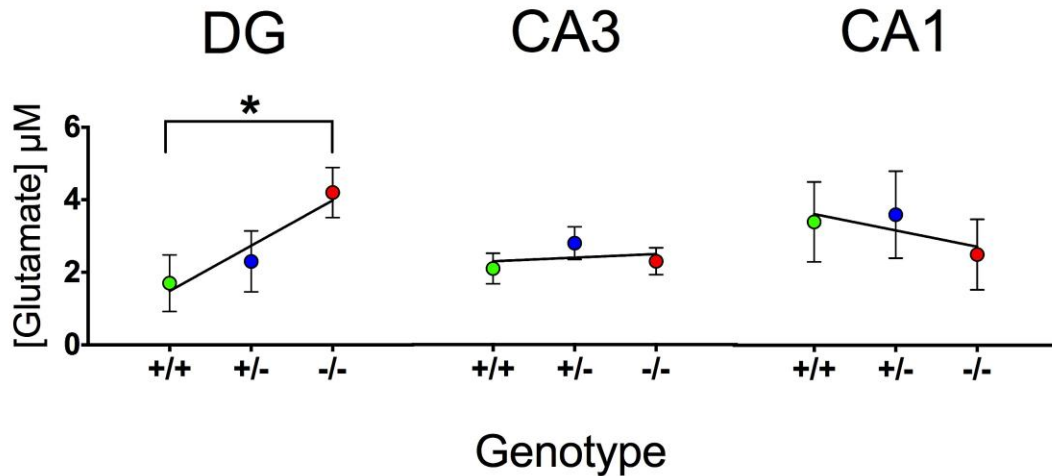


Figure 3.5 Amplitude of KCl-Evoked Glutamate Peaks is Larger in Tomosyn^{-/-} Mice Compared to Tomosyn^{+/+} Mice in the DG

A significant difference between Tom^{+/+} and Tom^{-/-} animals and a linear trend across genotype was observed in the DG in the amplitude of KCl-evoked glutamate peaks. Thus, a loss of tomosyn causes the maximum amplitude of an evoked glutamate peak to be larger in a given evoked event compared to wild-type mice. No statistical difference or linear trend was seen in the CA3 or CA1.

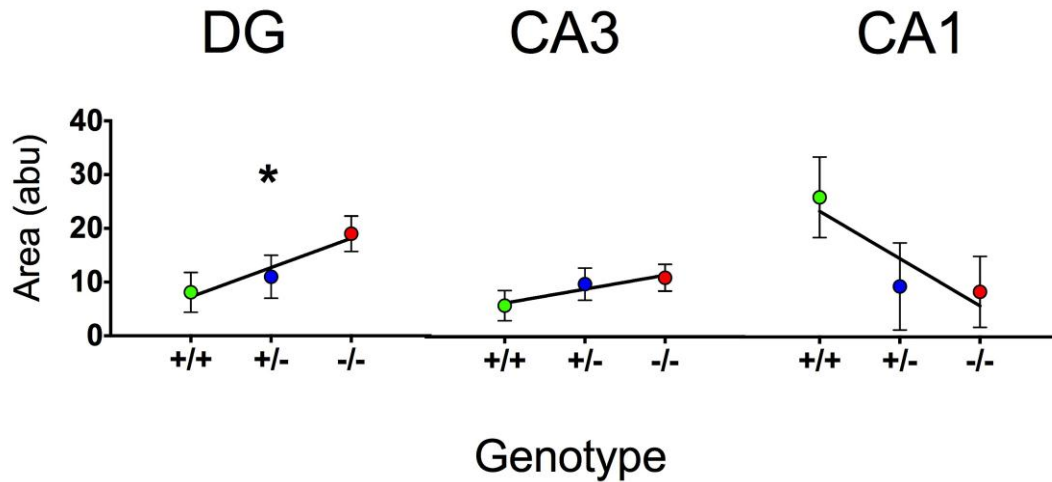
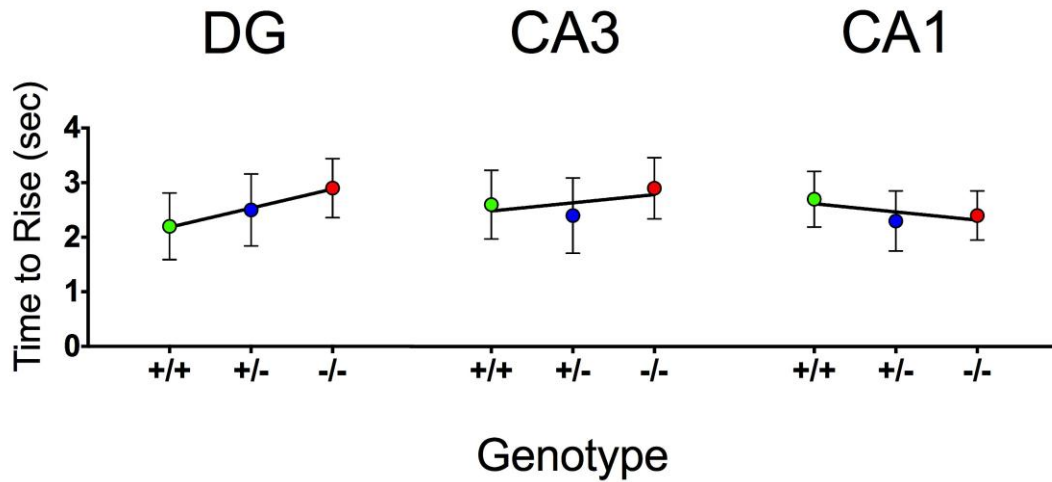


Figure 3.6 The Total Amount of Evoked Glutamate Release Increased as Tomosyn Decreased Across Genotype in the DG

No difference was seen between or within genotype in the area under KCl-evoked peaks. A linear trend was observed in this measure in the DG suggesting that as tomosyn decreases the total amount of glutamate released in a given evoked event increases.



3.7 KCl-Evoked Glutamate Release is Not Faster in Tomosyn^{-/-} Mice

No significant difference was seen between or within genotype in the time to rise of KCl-evoked glutamate peaks. No linear trend was seen in this measure across genotype. This lack of difference suggests that a loss of tomosyn does not affect how fast glutamate is released from neurons in the trisynaptic circuit.

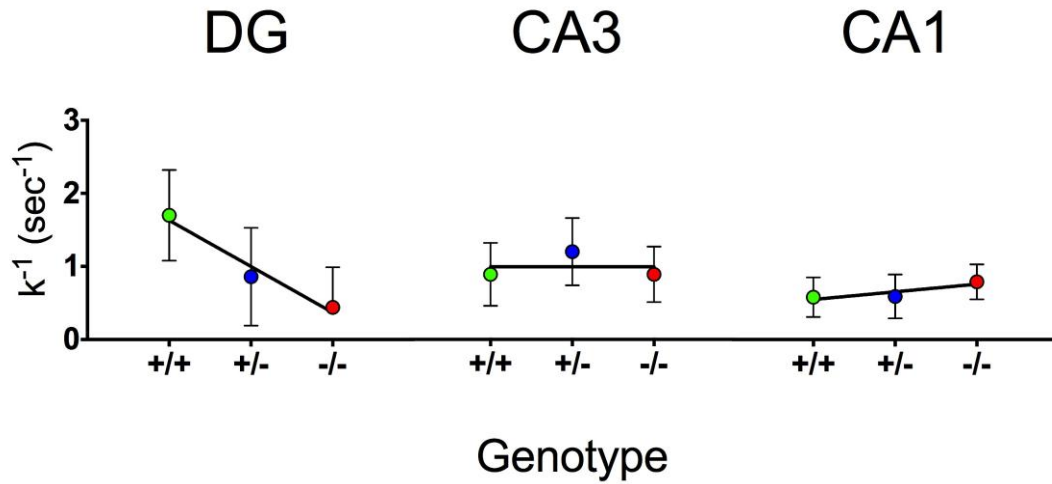


Figure 3.8 The Uptake of KCl-Evoked Glutamate Peaks is Not Faster in Tomosyn $^{-/-}$ Mice

No significant difference was seen between or within genotype in the decay rate of KCl-evoked glutamate peaks. No linear trend was observed in this measure across genotype. Taken together these results suggest that a loss of tomosyn does not affect the uptake rate of glutamate in the trisynaptic circuit.

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Publications

Abstracts

Batten SR, Matveeva E.A., Whiteheart S.W., Vanaman T.C., Glaser P.E.A., Slevin JT, Gerhardt GA (2013). Tomosyn Dysregulation Leads to Aberrant Glutamate Release in the Dentate Gyrus of the Hippocampus in a Mouse Model of Epileptogenesis. Presented by Batten SR at the Bluegrass Chapter for Neuroscience Spring Neuroscience Day in Lexington, KY.

Davis VA, Stephens ML, **Batten SR**, Price DA, Alcalá, R, Pomerleau F, Huettl P, Quintero JE, Slevin JT, Gerhardt GA (2012). Enzyme Based Microelectrode Arrays Offer Novel Insights into Mechanisms of Epilepsy. Presented by Davis VA at *In Vivo* Methods, Monitoring Molecules in Neuroscience in London, England.

Davis VA, **Batten SR**, Quintero JE, Pomerleau F, Huettl P, Gerhardt GA (2012). Measuring nitric oxide in the rat corpus cavernosum: can it be done? Presented by Davis VA and Batten SR at the Appalachian Health Summit Center for Clinical and Translational Science meeting in Lexington, KY.

Martin CA, Guenthner G, Charnigo R, **Batten SR**, Lile JA, Kelly TH (2011). Psychological symptoms and modafinil effects on smoking cessation. Presented by Martin CA at the international research symposium for The College on Problems of Drug Dependence.