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Jennifer Caldwell Wilhelm Date

The $GABA_A$ receptor is a critical part of the sensing machinery that triggers homeostatic plasticity of synaptic strength and intrinsic excitability.

By

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Doctor of Philosophy

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By

Jennifer Caldwell Wilhelm

B.S., Davidson College, 2001

Advisor: Peter Wenner, Ph.D.

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By Jennifer Caldwell Wilhelm

Generating and maintaining stability is a problem that neural networks must manage during development. Whereas Hebbian forms of plasticity allow for fast experience-dependent modification of synapses, these changes in synaptic strength can lead to the destabilization of neuronal firing rates if long-term potentiation and long-term depression are unconstrained. A new set of mechanisms, termed homeostatic plasticity, aids in the stabilization of network spiking activity by restraining the average amount of spiking activity within a cell or a network of cells within a set range. Experimentally homeostatic plasticity has been examined by pushing spiking activity to extremes; either all activity is blocked or activity is highly enhanced. Studies in multiple experimental systems have found that chronically reducing spiking activity in synaptically connected neurons triggers coordinated increases in excitatory synaptic strength as well as increases in intrinsic cellular excitability. These changes are thought to be homeostatic as they act to increase activity to recover normal levels. The mechanisms by which cells monitor spiking activity and trigger compensatory changes in synaptic strength are not well understood. Cells could monitor the average level of membrane depolarization or the activation of neurotransmitter receptors as a proxy for activity. Few studies have been able to separate the roles of membrane depolarization and neurotransmission. However, using the developing chick embryonic spinal cord, we are able to block either excitatory $GABA_A$ergic or glutamatergic transmission without significantly altering membrane depolarization. We found that blocking $GABA_A$ receptors, but not glutamate receptors, triggered compensatory increases in synaptic strength and cellular excitability similar to those demonstrated after activity block. This suggests a special role for the $GABA_A$ receptor as a critical part of the machinery that senses changes in activity and triggers compensatory mechanisms.

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CHAPTER ONE:

GENERAL INTRODUCTION

Creating balance between excitatory and inhibitory synapses in a network is critical to proper function because errors in this process can produce lifelong consequences. An excess of excitatory inputs can lead to seizures and neurotoxicity; an excess of inhibitory inputs can prevent network function (Ben-Ari, 2001). Generating and maintaining stability is a problem that neural networks must manage during development. Neurons receive input from many different synaptic partners, and these inputs can change dramatically throughout network development. Inputs are strengthened, weakened, or eliminated over time. It is important to have mechanisms in place that will allow neurons to integrate and adapt to the changing input while maintaining relative stability.

Historically, a large amount of research has focused on mechanisms such as long-term potentiation (LTP) and long-term depression (LTD) to explain experience-dependent modifications in synapses (Bi and Poo, 2001; Malinow and Malenka, 2002). LTP and LTD are considered Hebbian forms of plasticity in which correlated presynaptic and postsynaptic activity strengthens synapses, while uncorrelated activity weakens synapses. For example, correlated increases in firing rates in both the presynaptic and postsynaptic cells will strengthen the synaptic connection. However, these mechanisms are unlikely to account entirely for the ability of developing neurons to form stable networks (Turrigiano and Nelson, 2000). When a synapse is strengthened, the increased strength of the presynaptic neuron would drive the depolarization of the postsynaptic neuron more strongly, therefore creating a positive feedback loop in which the neuron could be strengthened indefinitely. The opposite could be true

for synapse weakening; any uncorrelated activity could cause the synapse to be weakened until the synapse was no longer functional. In fact, the positive feedback loop formed by Hebbian processes tends to destabilize postsynaptic firing rates. Mechanisms must exist to stabilize firing rates while permitting the strength of synapses to be modified (Turrigiano and Nelson, 2000; Davis and Bezprozvanny, 2001; Marder and Prinz, 2002; Burrone and Murthy, 2003).

Within the last decade or so, a new form of plasticity has been proposed that works to maintain stability within a network of neurons that are subject to Hebbian plasticity. Unlike Hebbian plasticity, this new type of plasticity, termed homeostatic plasticity, regulates the total synaptic strength of all neuronal synapses within a network while maintaining relative differences in the strength between synapses. Thus, homeostatic mechanisms can provide a complementary framework in which Hebbian plasticity can occur.

Homeostatic regulation of neuronal firing rates

The concept of homeostasis was coined by Cannon in 1932 (Cannon, 1932) and has come to encompass a wide range of physiological parameters, including body temperature and blood pressure. In general, homeostasis is the tendency to return to and remain within a set range. There is compelling evidence that neuronal activity patterns or levels of activity can be homeostatically regulated.

One of the first studies of the homeostatic regulation of neuronal activity was performed in the lobster stomatogastric ganglion (STG). In this study, Eve

Marder, Larry Abbott, and colleagues (Turrigiano et al., 1994) examined how cells of the STG responded to changes in synaptic and neuromodulatory inputs from other cells. Individual neurons were isolated from the STG and were placed in single-cell cultures. Electrophysiological recordings from these single cells showed little spiking activity after acute isolation (Figure 1.1). However, after 3-4 days *in vitro*, the individual cells began to demonstrate the stereotypic bursting properties similar to those recorded from cells in the intact STG. This recovery of activity is thought to be homeostatic because activity levels and patterns returned to normal. Further studies revealed that this recovery of spiking activity was mediated by changes in the densities of voltage-gated channels (Turrigiano et al., 1995; Marder and Prinz, 2002; Marder and Prinz, 2003; Marder and Goaillard, 2006). Providing several hours of rhythmically patterned depolarization or blocking calcium entry into the cell prevented these changes in channel density. These findings were among the first that suggested that activity perturbations in neurons could trigger processes that homeostatically regulated neuronal activity levels.

Several studies using cultured neurons from the central nervous system have shown homeostatic recovery of spiking activity following activity perturbations (Turrigiano et al., 1998; Burrone et al., 2002; Karmarkar and Buonomano, 2006). Blocking network activity for 2 days in cultured cortical networks using the sodium channel antagonist tetrodotoxin (TTX) produces an increase in cell spiking activity after washout of TTX. To test if this homeostatic process is bidirectional, Turrigiano and colleagues (Turrigiano et al., 1998)

increased spiking activity by blocking inhibitory transmission using the $GABA_A$ receptor antagonist bicuculline. After 2 days of bicuculline treatment, activity levels had returned to pre-treatment levels. This suggested that neuronal firing rates could be bidirectionally regulated to recover normal activity levels. A similar recovery of firing rates was seen in cultured hippocampal network when neurons were persistently hyperpolarized by the expression of an inwardly rectifying potassium channel (Kir). In this study, neuronal firing rates recovered to control levels even with continued expression of the hyperpolarizing channel (Burrone et al., 2002). Together, these findings suggest that perturbations to cell spiking activity (decreases or increases) can trigger mechanisms that act to recover normal activity levels.

Although studies have found many different ways by which neurons can adapt to activity perturbations, for clarity, I will mainly focus on homeostatic changes in quantal currents and intrinsic cellular excitability in mammalian central synapses. In this chapter I will first examine the mechanisms regulating the homeostatic changes in quantal currents followed by an examination of the ability of cells to alter voltage-gated channel densities to homeostatically maintain activity.

Homeostatic regulation of activity: scaling of quantal amplitudes

One mechanism for adjusting firing rates is the regulation of synaptic strength. In this dissertation changes in synaptic strength are defined as anything that will alter evoked synaptic current. The process of modifying the

strength of synapses in response to activity perturbations will be defined as homeostatic synaptic plasticity.

Several studies have blocked or significantly reduced spiking activity throughout an entire network. Although no homeostatic recovery of normal activity levels was observed, compensatory changes in synaptic strength were shown that acted in a direction to recover normal activity levels (Lissin et al., 1998; O'Brien et al., 1998; Turrigiano et al., 1998; Galante et al., 2001; Paradis et al., 2001; Kilman et al., 2002). In these studies, several different synaptic modifications were observed, including pre- and postsynaptic alterations.

The most prevalent modification observed was a compensatory change in the amplitude of miniature postsynaptic currents (mPSCs or quantal currents; Figure 1.2). Measuring mPSCs, which are evoked by the action potential-independent release of a single synaptic vesicle of neurotransmitter, allows for the quantification of the strength of a large number of synapses [reviewed in (Turrigiano and Nelson, 2004)]. Using whole-cell voltage clamp at the cell soma, mPSCs resulting from the release of neurotransmitter from multiple presynaptic partners can be measured. This allows for the analysis of a large number of synapses at the same time. By measuring mPSCs rather than evoked synaptic potentials, one can measure the smallest changes in the strength of many different synapses. When measuring mPSCs in cultured cells, many different studies have found that activity reductions increased the amplitudes of excitatory [alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA-) and N-methyl-D-aspartate (NMDA-)] mPSCs and decreased the amplitude of inhibitory

(GABA-) mPSCs. Conversely, when network activity was increased excitatory mPSC amplitude was decreased, again in a compensatory fashion. Therefore, when activity is chronically perturbed, cells or networks can alter mPSC amplitude to compensate for the perturbation. These activity-dependent changes in quantal amplitude have been shown in many types of neurons, including neocortical (Turrigiano et al., 1998), hippocampal (Lissin et al., 1998), and spinal (O'Brien et al., 1998) neurons.

Generally, these activity-dependent increases or decreases in mPSC amplitude occurred across the entire distribution of mPSC amplitudes (Figure 1.2). For example, plotting AMPA-mPSC amplitudes as a cumulative histogram shows that the entire distribution of AMPA-mPSC amplitudes are increased (reduced activity) or decreased (increased activity). Multiplying each value in the distribution by a certain factor will scale the distribution up or down such that it overlays the control distribution. This multiplication process, termed synaptic scaling, suggests that activity perturbations trigger a mechanism that multiplicatively adjusts the strength of all synapses throughout the network (Turrigiano et al., 1998).

Synaptic scaling is thought to be a non-Hebbian form of plasticity because it alters synapses globally and does not depend on correlations between pre- and postsynaptic activity (Abbott and Nelson, 2000). Synaptic scaling allows total synaptic strength to be adjusted while the relative differences in the strength of individual synapses are maintained. Thus, this provides a way for global

alterations in the level of excitation within the network while preserving relative differences in the strength of synapses.

Most studies of synaptic scaling have shown that 24-48 hours of activity block were necessary to trigger compensatory changes. However, one recent study has demonstrated that synaptic scaling through a change in the density of postsynaptic glutamate receptors can be triggered within 4 hours of activity block (Ibata et al., 2008). These unusual findings appear to be dependent on the conditions of the cultures used for the recordings. It is unclear if these changes in quantal amplitude are mediated by the same mechanisms that underlie changes in quantal amplitude after longer-term activity block. As this phenomenon has only been shown in one study, I will primarily discuss the homeostatic mechanisms that are likely to be engaged after 48 hours of activity block.

Homeostatic synaptic scaling: possible mechanisms

Increases in glutamatergic mPSC amplitude could be mediated by several changes, including increases in the presynaptic filling of vesicles with transmitter, decreases in the reuptake of transmitter, or increases in the number of postsynaptic receptors. Although there are multiple changes that can produce increased excitatory quantal amplitude, I will focus on changes for which there is experimental evidence.

Changes in postsynaptic receptors at least partially underlie synaptic scaling. Studies have found that chronic activity block produces an increase in

the responsiveness of postsynaptic membrane to glutamate agonists (O'Brien et al., 1998; Turrigiano et al., 1998), an increase in the half-life of AMPA receptors (O'Brien et al., 1998), an increase in the number of channels that open in response to glutamate application (Watt et al., 2000), and an increase in the number of synaptic AMPA receptors (Lissin et al., 1998; O'Brien et al., 1998; Turrigiano et al., 1998; Wierenga et al., 2006). The increase in number of channels has been best described. Using quantitative immunoflourescence, several studies have shown bidirectional changes in the accumulation of post-synaptic AMPA receptors after activity perturbations (Lissin et al., 1998; O'Brien et al., 1998; Turrigiano et al., 1998; Wierenga et al., 2005). The subunit composition of the AMPA receptors that are accumulated appears to depend on the type of neuronal cell examined. Within spinal and neocortical cultures, AMPA receptors containing the glutamate receptor 1 (GluR1) or glutamate receptor 2 (GluR2) subunits are increased proportionally after activity blockade (O'Brien et al., 1998; Wierenga et al., 2005). However in hippocampal neurons activity block selectively increases GluR1-containing AMPA receptors without affecting the number of GluR2-containing AMPA receptors (Ju et al., 2004; Thiagarajan et al., 2005; Sutton et al., 2006). These changes in receptor accumulation are likely due to an activity-dependent decrease in the turnover rate of synaptic AMPA receptors (O'Brien et al., 1998) after activity block. However, differential scaling of receptor insertion by activity perturbations could also produce multiplicative scaling (Turrigiano and Nelson, 1998). The involvement of NMDA receptors in homeostatic scaling is controversial. Most studies have only shown changes in

AMPA receptors; however, a few studies have suggested that NMDA receptors may be homeostatically regulated. One study in neocortical cultures has shown that activity block can increase the expression of both AMPA and NMDA receptors in a proportional manner such that the ratio of AMPA and NMDA receptors is maintained (Watt et al., 2000). A separate study in hippocampal cultures found that activity block homeostatically increases the clustering of synaptic NMDA receptors (Rao and Craig, 1997). This increased accumulation of NMDA receptors could occur through the activity-dependent alternative splicing of the NMDA receptor 1 (NR1) subunit, a subunit that regulates export from the endoplasmic reticulum and accumulation at the surface (Mu et al., 2003).

Presynaptic changes could also mediate synaptic scaling. An increase in the concentration of transmitter in individual vesicles has been shown in activity-reduced cultured cells (De Gois et al., 2005; Wilson et al., 2005; Erickson et al., 2006; Hartman et al., 2006). Chronic activity block increases the expression of vesicular glutamate transporter 1 (VGLUT1); increasing activity decreases VGLUT1 expression (De Gois et al., 2005; Erickson et al., 2006). Changes in the vesicular glutamate transporter levels could alter the amount of glutamate packaged into synaptic vesicles, thereby altering quantal amplitude (Wilson et al., 2005; Moechars et al., 2006).

Although I have focused on excitatory synapses, it is important to note that inhibitory synapses also are homeostatically regulated. Activity block reduces the amplitude of inhibitory quantal currents in cortical cultures (Kilman et al.,

2002). The mechanisms underlying the changes in inhibitory synapses are not well understood.

Homeostatic synaptic scaling: potential signaling molecules

Several molecules have been identified as possible mediators of synaptic homeostasis including the trophic factor, brain-derived neurotrophic factor (BDNF), the effector immediate-early gene product, Arc, the immune factor, cytokine tumor-necrosis factor-alpha (TNF-alpha), and the synaptic cell adhesion molecule, beta-3 integrin (Figures 1.3 and 1.4). The production or regulation of these molecules has been shown to alter the accumulation of AMPA receptors (Rutherford et al., 1998; Beattie et al., 2002; Stellwagen et al., 2005; Shepherd et al., 2006; Stellwagen and Malenka, 2006; Cingolani et al., 2008). Blocking or enhancing the signaling of these molecules can mimic the effects of activity perturbations, suggesting that these factors are involved in the homeostatic quantal scaling in cultured neurons (Rutherford et al., 1998; Beattie et al., 2002; Stellwagen and Malenka, 2006; Cingolani et al., 2008). Whether these molecules are involved in triggering synaptic scaling *in vivo* is unknown.

Studies in cultured networks have demonstrated that BDNF can alter both pre- and postsynaptic properties of GABAergic and glutamatergic synapses in response to changes in levels of network activity. BDNF production and release is regulated by activity; chronic increases or decreases in network activity levels produce changes in BDNF release in cultured cortical neurons (Rutherford et al., 1998). These alterations in BDNF signaling have been linked to changes in

synaptic strength in culture (Rutherford et al., 1998; Wardle and Poo, 2003; Swanwick et al., 2006). Exogenous BDNF application to activity blocked cultured neurons prevents the expected increases in quantal amplitude of excitatory synapses (Rutherford et al., 1998). Blocking the BDNF receptor, neurotrophic tyrosine kinase receptor type 2 (TrkB) reproduced changes in quantal amplitude that would be expected after activity blockade (Rutherford et al., 1998). Interestingly, in cultured hippocampal networks, activity deprivation reduced inhibitory quantal amplitude, and here exogenous BDNF, signaling through TrkB receptors, restored the normal inhibitory quantal amplitudes (Lessman et al., 1994; Li et al., 1998; Rutherford et al., 1998; Tyler and Pozzo-Miller, 2001; Obrietan et al., 2002). These findings suggest that during activity blockade, reduced BDNF signaling mediates compensatory changes in synaptic strength of excitatory and inhibitory synapses *in vitro*.

The activity-dependent expression of the effector immediate-early gene product Arc has been implicated in the homeostatic accumulation and regulation of postsynaptic AMPA receptors. Arc has been shown to mediate the accumulation of AMPA receptors via the activation of an endocytic pathway involving the proteins endophilin 2/3 and dynamin (Chowdhury et al., 2006). Overexpression of Arc blocks the upregulation of AMPA receptors; whereas loss of Arc increases AMPA-mPSC amplitude and AMPA receptor surface expression (Shepherd et al., 2006). Thus, activity block likely reduces Arc signaling, thereby reducing the endocytosis of AMPA receptors and increasing the accumulation of membrane bound AMPA receptors. Together, these findings support the idea

that Arc signaling is a critical component to AMPA receptor mediated homeostatic synaptic scaling.

TNF-alpha also has been shown to increase the surface expression of AMPA receptors in hippocampal cultures (Beattie et al., 2002; Stellwagen et al., 2005; Stellwagen and Malenka, 2006). Stellwagen and Malenka (Stellwagen and Malenka, 2006) demonstrated that bath application of TNF-alpha is sufficient to increase the surface expression of AMPA receptors as well as increase the amplitude of AMPA-mPSCs. They went on to show that TTX can induce the upward scaling of AMPA-mPSCs through a glial-dependent release of TNF-alpha. In their model, Stellwagen and Malenka suggest that reducing spiking activity with TTX reduces the release of glutamate into the extracellular milleu. A reduction in glutamate binding to receptors on glia triggers an increase in the release of TNF-alpha. Then TNF-alpha can alter the expression of AMPA receptors via currently unknown mechanisms.

A recent study has implicated the synaptic cell adhesion molecule beta-3 integrin in the homeostatic accumulation of AMPA receptors (Cingolani et al., 2008). Integrins are heterodimeric transmembrane receptors for extracellular matrix proteins and counterreceptors on adjacent cells. These receptors have been shown to be important for several aspects of neuronal plasticity; this is the first study to demonstrate a role for an integrin in homeostatic synaptic plasticity. In this study, activity block increases the postsynaptic surface expression of beta-3 integrins, which produces an increase in the accumulation of surface GluR2-containing AMPA receptors by reducing receptor endocytosis. Interestingly,

beta-3 surface expression is increased to a larger extent when cells are treated with TNF-alpha than when treated with TTX to block activity. This suggests that TNF-alpha may mediate increases in quantal amplitude by increasing the surface expression of beta-3 integrins.

It is not completely clear how these signaling molecules work to trigger changes in quantal amplitude. Activity block likely reduces BDNF release and Arc signaling and increases TNF-alpha release and TNF-alpha induced surface expression of beta-3 integrins (Figure 1.4). It is unknown if all of these molecules are activated by activity reduction in all cells or if these molecules activated in a cell-specific manner. Future studies are necessary to determine the roles that each of these molecules and current unidentified molecules play in translating activity perturbations into alterations in synaptic strength.

Homeostatic regulation of activity: changes in mPSC frequency

The majority of studies of homeostatic synaptic plasticity in central synapses have focused on compensatory changes in quantal amplitude (O'Brien et al., 1998; Turrigiano et al., 1998; Watt et al., 2000; Kilman et al., 2002); however, several studies have found evidence for activity-dependent changes in the frequency of quantal currents (Murthy et al., 2001; Burrone et al., 2002; Thiagarajan et al., 2002; Thiagarajan et al., 2005; Wierenga et al., 2006). These changes in frequency can occur alone (Burrone et al., 2002; Wierenga et al., 2006) or in conjunction with changes in quantal amplitude (Thiagarajan et al., 2002; Thiagarajan et al., 2005; Wierenga et al., 2006). Increasing the frequency

of quantal currents after activity block could contribute to the recovery of activity levels by strengthening synapses.

Changes in mPSC frequency could be due to a change in the probability of release of vesicles, a change in the total number of synaptic boutons, or a change in the number of functional release sites per synaptic bouton. The total number of synaptic boutons does not appear to be affected by activity block (Burrone et al., 2002; Kilman et al., 2002). One study has shown that synapses can increase in size by approximately 30% and that this increase also corresponds with an increase an in the number of docked vesicles in response to 2 days of activity block (Murthy et al., 2001). Studies of activity block in hippocampal cultures have suggested that the number of docked vesicles per active zone is increased after activity block as is the size of the recycling vesicle pool (Murthy et al., 2001). In addition, studies at the mouse neuromuscular junction have shown homeostatic changes in the probability of release of vesicles that are mediated by changes in calcium entry through P/Q-type calcium channels (Wang et al., 2004). Because data about changes in mPSC frequency have been collected in multiple different systems at different stages of development, it is unclear which, if not all, mechanisms are engaged to alter mPSC frequency after activity perturbations.

The discrepancy in the reporting of changes in the frequency of quantal currents could be due to differences in culture conditions. A recent study demonstrated that activity block in sister cultures of hippocampal or cortical neurons produced changes in mPSC amplitude but not mPSC frequency if *in*

vitro less than 2.5 weeks; however, activity block of cultures maintained *in vitro* longer than 3 weeks produced changes in both mPSC amplitude and mPSC frequency (Wierenga et al., 2006). It is unclear whether the *in vitro* environment alters the response of the cultures to activity block or if a difference in maturation of the networks is responsible. It is also possible that intrinsic differences among distinct neuronal types causes different reactions to similar manipulations (Burrone and Murthy, 2003).

Possible sensors for activity

Cells or networks of cells appear to try to maintain a specific range of cell spiking activity; however, the sensors that detect levels of activity are unclear (Figures 1.3 and 1.4). Sensors report the difference between the set range of the system and the actual output of the cell. There must be at least one, and possibly multiple, sensors that monitor cell and/or network activity and trigger changes in synaptic strength in response to activity perturbations (Davis, 2006; Rich and Wenner, 2007). Although the homeostatic synaptic plasticity field has focused on average postsynaptic membrane depolarization as the likely sensor for changes in synaptic strength, few studies have been able to differentiate between reductions in membrane depolarization and reductions in postsynaptic receptor activation. Traditionally homeostatic processes are examined by blocking activity using the sodium channel blocker, TTX, or the AMPA receptor antagonist, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX). The addition of TTX blocks sodium channels, which directly blocks action potentials and membrane

depolarization, but TTX also indirectly reduces neurotransmitter receptor activation by reducing the action potential dependent release of neurotransmitter. Blocking AMPA receptors directly using CNQX indirectly reduces cell spiking by reducing cellular depolarization. To properly examine the nature of the sensor, one would need to reduce spiking without altering transmission or block transmission without altering spiking.

Several studies have suggested that changes in postsynaptic receptor activation, rather than postsynaptic membrane depolarization, may be important in triggering synaptic scaling. Murthy and colleagues (Burrone et al., 2002) expressed an inwardly rectifying potassium channel (Kir) in individual neurons to selectively hyperpolarize neurons within the network. Recordings from the transfected neurons showed no change in quantal amplitude, which is opposite of what be expected if reduction in membrane depolarization triggers increases in quantal amplitude. This finding is inconsistent with the idea that membrane depolarization alone triggers changes in quantal amplitude. However, it is consistent with the idea that postsynaptic receptor activation is part of the sensing machinery. When activity is blocked in an individual cell, neurotransmission through that cell's postsynaptic receptors remains intact because inputs from other cells in the network are still active. Similar results have also been suggested in studies *in vivo*. When activity in a subpopulation of presynaptic muscle spindle afferents is blocked, the synaptic strength onto motoneurons of the blocked afferents, but not other afferents, is increased within days (Gallego et al., 1979; Manabe et al., 1989; Webb and Cope, 1992).

Because a subpopulation of afferents, rather than all inputs, was blocked, the activity of the postsynaptic motoneuron remained relatively normal. These findings suggest that reducing presynaptic activity and neurotransmission is able to trigger homeostatic changes in synaptic strength in the absence of changes in postsynaptic membrane depolarization. Thus, the effects of activity blockade on quantal amplitude may be due to reducing synaptic transmission of all of a cell's inputs. In the experiments described in chapter 2, we will test the hypothesis that chronic blockade of GABA and AMPA receptors triggers changes in synaptic strength observed after activity blockade.

One study has examined the effects of directly depolarizing neurons by increasing extracellular potassium while blocking AMPA and NMDA receptors to increase cell-spiking activity. This method triggers increases in inhibitory synaptic strength in a compensatory manner; thus suggesting that postsynaptic membrane depolarization may be important for triggering homeostatic changes in synaptic strength in response to increased activity. However, different mechanisms are engaged when activity is increased versus decreased (Leslie et al., 2001). The question still remains whether decreased spiking activity or reduced postsynaptic receptor activation triggers changes in synaptic strength during activity block.

Differences between homeostatic and Hebbian synaptic plasticities

Although both Hebbian and homeostatic synaptic plasticities adjust the strength of synapses, there are many differences between the two. Hebbian plasticity is triggered by associative changes in activity in pre- and postsynaptic

neurons. This type of plasticity occurs relatively quickly and strengthens synapses with correlated activity in a synapse specific manner. Homeostatic synaptic plasticity is a slower process, requiring hours to days to occur [(Turrigiano and Nelson, 2004) but see (Ibata et al., 2008)]. This is an important feature as it allows neurons to integrate activity over time and ignores changes in response to fast activity fluctuations. Chronic activity perturbations trigger a homeostatic process in which the strength of all synapses, rather than specific, individual synapses, are scaled up or down by the same factor (Turrigiano, 1999). This allows for networks to maintain any relative differences in inputs, such as those created by the Hebbian processes LTP and LTD. The mechanisms by which Hebbian and homeostatic synaptic plasticity occur are somewhat different. Unlike most forms of Hebbian plasticity, homeostatic synaptic plasticity appears to be independent of NMDA receptor activation. Blocking AMPA receptor signaling or blocking spiking with TTX can trigger homeostatic increases in excitatory synaptic strength (Turrigiano and Nelson, 2004). However, chronically blocking NMDA receptor signaling alone does not alter synaptic strength [(Turrigiano et al., 1998; Watt et al., 2000; Leslie et al., 2001), but see (Lissin et al., 1998)]. While both Hebbian and homeostatic plasticity affect the accumulation of glutamate receptors, these changes occur in opposite directions. Acute increases in activity lead to the rapid insertion of glutamate receptors in LTP, but reduced activity increases receptor accumulation over longer timescales in homeostatic synaptic scaling [(Turrigiano and Nelson, 2004) but see (Ibata et al., 2008)].

Homeostatic plasticity: homeostatic changes in cellular excitability

The activity of a neuron is likely achieved through the combined influences of synaptic inputs and ion channels (Marder and Prinz, 2002; Marder and Prinz, 2003). Thus, it is possible that blocking activity can adjust both the strength of synaptic connections and cellular excitability (by altering the density of ion channels). Several studies in multiple different model systems (including hippocampal culture, neocortical culture, and STG) have demonstrated that intrinsic electrical properties of individual neurons can be regulated by activity (Brodie et al., 1989; Franklin et al., 1992; Turrigiano et al., 1994; Li et al., 1996; Marder et al., 1996; Desai et al., 1999c; Desai et al., 1999a; Aizenman and Linden, 2000; Armano et al., 2000; Ganguly et al., 2000; Nick and Ribera, 2000). These properties are mainly mediated by a neuron's distribution of ion channels (sodium channels, calcium channels, potassium channels, chloride channels). Modifying the magnitude and distribution of ion channels can affect neuronal excitability (Desai et al., 1999c), synaptic integration (Storm, 1988; LeMasson et al., 1993; Marder et al., 1996), and the pattern and rate of firing (Turrigiano et al., 1994; Turrigiano et al., 1995).

Pharmacological block of spiking activity for 2 days in cultured pyramidal neurons of the rat visual cortex triggers a significant increase in intrinsic cellular excitability (Desai et al., 1999a). In these experiments, neurons responded more rapidly to current injection after activity block, with the average slope of a plot of firing frequency versus current injection almost doubling. This suggests that the activity-deprived neurons were more sensitive to their inputs. These changes in

excitability appeared to be produced by coordinated changes in the densities of voltage-gated sodium and potassium currents. Activity block increased the amplitude of sodium currents, decreased the amplitude of persistent potassium currents (such as delayed rectifying K^+ currents and Ca^{2+}-activated K^+ channels), while leaving fast-inactivating potassium current (A-type K^+ currents) and calcium currents intact. The voltage dependence of the currents was not affected by activity block suggesting that the density of channels is being regulated by activity rather than properties of individual channels. It is also important to note that different currents were altered in different directions. Rather than increasing all currents, the balance of depolarizing and hyperpolarizing currents was differentially regulated. These findings are similar to those found in earlier studies in the STG in which STG neurons isolated from synaptic and modulatory inputs coordinately modify their ionic conductances to recover normal firing patterns (LeMasson et al., 1993).

The mechanisms that translate chronic changes in activity into changes in cellular excitability are not clear, but BDNF and intracellular calcium signaling have been implicated. One study found that blocking BDNF signaling though the TrkB receptor could trigger increases in cellular excitability similar to those triggered after activity block (Desai et al., 1999b). The addition of exogenous BDNF prevented activity-dependent increases in cellular excitability. This finding is interesting because BDNF has been shown to trigger changes in quantal amplitude in a similar manner (Rutherford et al., 1997; Rutherford et al., 1998). Intracellular calcium signaling is a good candidate for translating changes in

activity because the rate of calcium entry into a neuron has been shown to be correlated to the neuron's electrical activity (Ross, 1989). Additionally, changes in intracellular calcium have been shown to be associated with modifications of channel densities (Linsdell and Moody, 1995).

Spontaneous network activity in developing systems

During development neural circuits face multiple challenges in maintaining network stability. Many developing neural circuits in the central nervous system exhibit spontaneous waves of network activity. The role of this activity in regulating synaptic strength and cellular excitability is not clear. However, a recent study has suggested that reductions in spontaneous network activity (SNA) in the developing spinal cord are capable of triggering homeostatic increases in excitatory synaptic strength similar to those triggered after activity block in cultured networks (Gonzalez-Islas and Wenner, 2006). Further experiments are necessary to better understand the relationship between SNA and homeostatic plasticity *in vivo*.

Several distinct types of spontaneous activity are produced both before and after the development of chemical synaptic networks [reviewed in (O'Donovan et al., 1998a; O'Donovan, 1999; Ben-Ari, 2001). Spontaneous activity can emerge early during neural development prior to the formation of synaptic networks. This activity, produced by calcium transients that are coordinated in groups of cells by gap junctions, appears to be associated with neurite extension and outgrowth. SNA emerges once chemical synaptic

networks form. SNA has been described in most developing circuits shortly after synaptic connections first form, including the retina, hippocampus, and spinal cord (Gummer and Mark, 1994; Fortin et al., 1995; Itaya et al., 1995; Lippe, 1995; Feller, 1999; Ho and Waite, 1999; O'Donovan, 1999; Wong, 1999; Ben-Ari, 2001). In the spinal cord, SNA is known to be important for many aspects of development, including motoneuron axonal path finding (Hanson and Landmesser, 2004), proper muscle and joint development (Ruano-Gil et al., 1978; Toutant et al., 1979; Roufa and Martonosi, 1981; Persson, 1983; Hall and Herring, 1990; Jarvis et al., 1996), and regulation of synaptic strength [(Gonzalez-Islas and Wenner, 2006) and Chapter 2]. Although SNA *in vivo* activates motoneurons and generates limb movements, SNA is likely resultant from different mechanisms than those that govern locomotor-like activity and does not depend on pacemaker or oscillator neurons [reviewed in (O'Donovan et al., 1998a)]. In this dissertation, I will focus on SNA in the developing spinal cord.

SNA is characterized by bursts or episodes of spiking activity that are separated by longer periods of quiescence. These episodes are a consequence of the highly excitable nature of a synaptic circuit, in which the main neurotransmitters (GABA and glutamate) are depolarizing and excitatory (Ben-Ari, 2001). During the episode, spinal neurons fire rhythmically, with alternation of discharge in flexors and extensors (Landmesser and O'Donovan, 1984; O'Donovan, 1989). After an episode of activity, spinal neurons are transiently hyperpolarized, decreasing network excitability (Chub and O'Donovan, 2001).

Network excitability gradually recovers during the period between episodes (Tabak et al., 2001). Eventually, enough neurons become depolarized to cause spiking activity throughout the network, thereby initiating the next episode (O'Donovan et al., 1998a). The combination of the hyper-excitable network and the activity-dependent depression of neuronal and network excitability allows for the rhythmic, episodic nature of SNA (O'Donovan et al., 1998a; O'Donovan et al., 1998b; O'Donovan, 1999). The mechanisms that are responsible for the generation of activity-dependent depression are not completely understood, however, several studies have suggested a role for $GABA_A$ mediated chloride signaling [reviewed in (O'Donovan et al., 1998a)].

Although the transmitter GABA is inhibitory and hyperpolarizing in mature systems, GABA is depolarizing because intracellular chloride is high in early development [Figure 1.5; (Ben-Ari, 2002; Ben-Ari et al., 2007)]. $GABA_A$ receptors are composed of a pentameric assembly of subunits that form an ion channel that is predominately permeable to chloride (Ben-Ari, 2002; Ben-Ari et al., 2007). During development, GABA binding to the $GABA_A$ receptor opens the channel allowing chloride to rush out of the cell, thereby reducing the polarization of the cell. During the episode, a large amount of GABA is released, causing a significant loss of intracellular chloride. This loss of chloride changes the chloride reversal potential and weakens the driving force for chloride to leave the cell (Chub and O'Donovan, 1998; O'Donovan, 1999; Chub and O'Donovan, 2001; Marchetti et al., 2005). Thus just after the end of an episode, the amplitudes of GABAergic synaptic currents are depressed compared to the amplitudes before

the episode began [(Chub and O'Donovan, 2001), Figures 1.6 and 2.9]. The amplitude of the synaptic currents recovers during the inter-episode interval due to the activation of a Na^+-K^+-$2Cl^-$ co-transporter (NKCC1) that pumps chloride back into the cells. This pump accumulates chloride, thereby increasing the driving force for chloride and increasing the amplitude of $GABA_A$-mediated currents.

As the networks develop, SNA is reduced in part to the change in GABA from an excitatory, depolarizing transmitter to an inhibitory, hyperpolarizing transmitter (Figure 1.5). This change occurs due to a decrease in intracellular chloride levels. Later in development, the chloride accumulator NKCC1 is down regulated as the chloride extruder, KCC2, is up regulated. This change in chloride pumps causes intracellular chloride levels to be reduced and chloride currents thus become hyperpolarizing and inhibitory (Ben-Ari et al., 2007). This occurs in the developing chick spinal cord at approximately embryonic day 15 (Xu et al., 2005).

SNA after neurotransmitter block in the *in vitro* chick spinal cord.

The chick embryo is a powerful model that we can use to functionally assay the strength of synaptic connections following blockade of neurotransmission in the intact embryo without the complications of maternal effects. Unlike other studies of homeostatic synaptic plasticity, we are able to examine the effects of chronic blockade of GABAergic or glutamatergic transmission while leaving SNA relatively unaltered in an intact developing

network. As both GABA and glutamate are depolarizing during early development, blocking either $GABA_A$ or AMPA receptors only reduces SNA briefly. This recovery of SNA has been shown in the *in vitro* spinal cord by monitoring spontaneous bursts of motoneuron activity through suction electrodes attached to intact muscle nerves (Chub and O'Donovan, 1998). Bath application of bicuculline to block $GABA_A$ receptors or CNQX to block AMPA receptors transiently blocks SNA, but SNA recovers within one hour of antagonist application. Once SNA recovers, the levels and patterns of activity appear relatively normal. The recovered SNA is now generated by the unblocked neurotransmitter system.

In the case of AMPA receptor blockade, the recovered SNA is driven by the remaining unblocked GABAergic transmission (Chub and O'Donovan, 1998; Tabak et al., 2001). This compensation occurs within minutes by short-term alterations in chloride pumps that increase the driving force for chloride. The increase in the loading of intracellular chloride increases the driving force for efflux of chloride through unblocked $GABA_A$ receptors, therefore increasing the amplitude of GABAergic currents. These short-term changes can now compensate for the loss of glutamatergic neurotransmission (Chub and O'Donovan, 1998; Tabak et al., 2001).

Episodes of SNA occur after $GABA_A$ receptor block, but the mechanisms underlying the recovery of SNA are less clear. In an untreated spinal network, SNA is generated in part by $GABA_A$ mediated chloride signaling (Chub and O'Donovan, 1998; Tabak et al., 2001). The extrusion of chloride by the activation

of $GABA_A$ receptors during the episode reduces the driving force for chloride, thereby reducing $GABA_A$ mediated currents after the episode (Figure 1.6). This post-episode depression of GABAergic currents helps produce the rhythmic pattern of SNA. When $GABA_A$ receptors are blocked *in vitro*, SNA can occur albeit less regularly, suggesting that other mechanisms must be responsible for the generation of activity. One study has suggested that an increase in the strength of glutamatergic synapses could mediate the recovery of SNA after $GABA_A$ receptor block (Tabak et al., 2001).

SNA and homeostatic synaptic plasticity.

Recently the Wenner lab demonstrated that chronically reducing SNA *in ovo* triggers compensatory increases in excitatory synaptic strength (Gonzalez-Islas and Wenner, 2006). Infusion of the sodium channel blocker lidocaine onto the chorioallantoic membrane of the embryonic chick *in ovo* reduced SNA for two days. Whole cell voltage clamp recordings from motoneurons in the isolated chick spinal cord revealed that excitatory GABAergic and AMPAergic quantal currents were increased in embryos treated for 2 days with lidocaine. These increases in quantal strength acted in a manner to compensate for the activity reduction and to recover normal activity levels. This study was one of the first to demonstrate that the process of homeostatic synaptic plasticity can occur after activity perturbations in an *in vivo* vertebrate system. However, similar to studies of homeostatic synaptic plasticity in cultured networks, activity block *in ovo* also reduced the release of neurotransmitter. The reduction in neurotransmission

could be critical to triggering increases in synaptic strength after activity reduction.

Objectives of this dissertation

The factors that trigger compensatory changes in quantal amplitude are unknown. In this dissertation, we will test the importance of transmission through GABA and glutamate receptors in triggering changes in quantal amplitude as well as other aspects of synaptic strength in the living embryo. Further, we will assess whether activity or neurotransmitter block *in ovo* triggers homeostatic changes in cellular excitability that could, in addition to changes in synaptic strength, help the cell be more responsive to synaptic inputs and recover normal activity levels. Understanding the mechanisms that drive homeostatic control of network excitability provides insight into the impact of perturbations that can occur during development. Clinically, understanding the effects of reducing fetal movements is critical. Drugs prescribed to pregnant women, such as methadone, can drastically reduce fetal movements that can cause developmental problems (Wouldes et al., 2004). Once we better understand the effects of altering SNA, we will be in a better position to help treat and prevent potential drug-induced developmental delays and disorders.

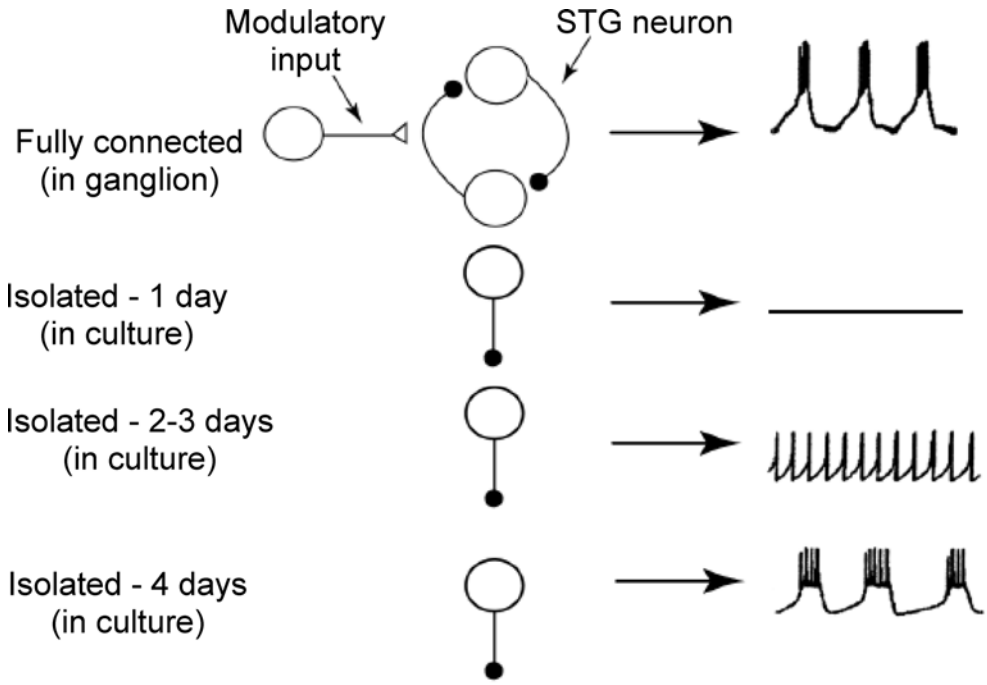

Figure 1.1. Isolated stomatogastric (STG) neurons can produce rhythmic bursting *in vitro*. Recurrently connected STG neurons in the intact ganglion, which normally receive synaptic and neuromdulatory input from neighboring neurons, have rhythmic bursting patterns of action potentials. The removal of a single neuron from the surrounding neuromodulatory and synaptic inputs causes the cell to cease firing action potentials. However, after 1 day in culture, the isolated neuron begins to tonically fire action potentials. After 4 days in culture, isolated neurons exhibit a rhythmic pattern of bursting similar to that expressed by neurons in the intact ganglion. These findings suggest that single neurons have the ability to adjust to loss of inputs and recover normal patterns of spiking activity. Figure modified from: (Turrigiano, 1999)

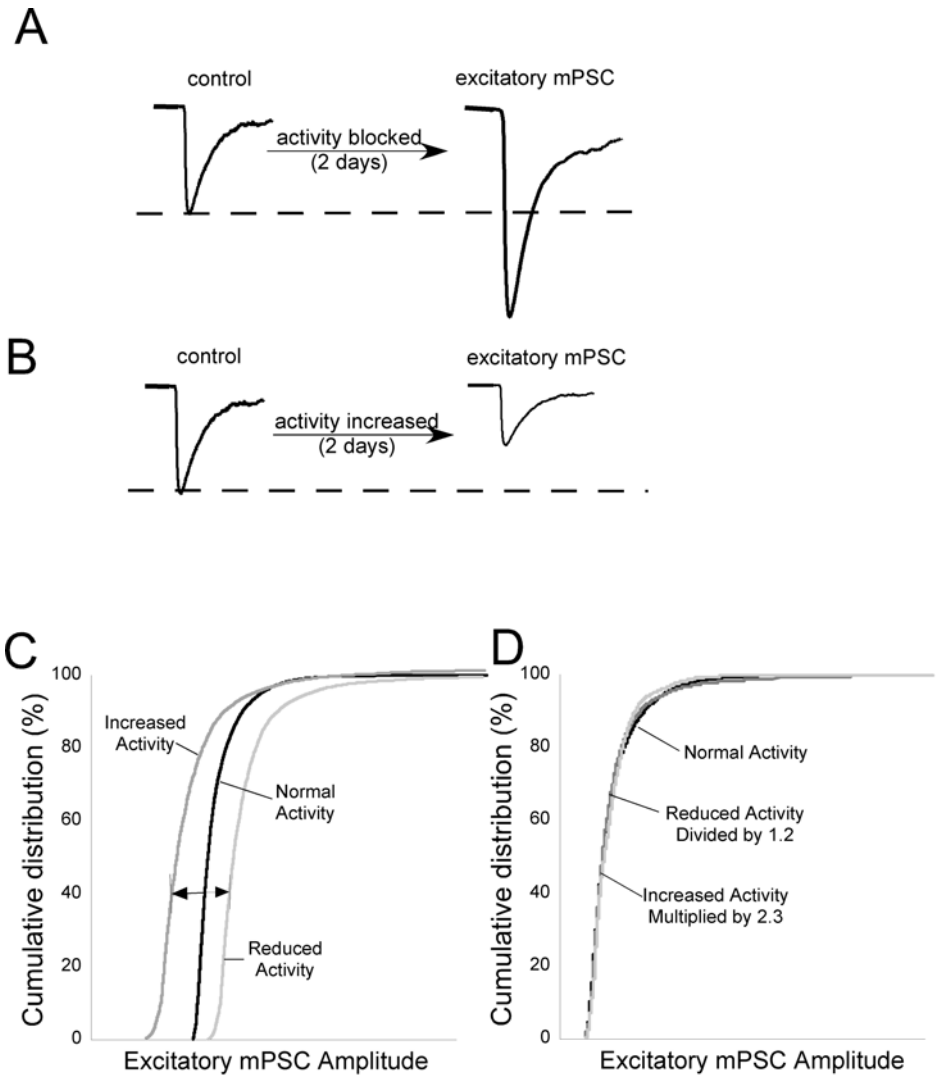

Figure 1.2. Alterations to network activity produces bidirectional compensatory changes in quantal amplitude. (A) Blocking activity for 2 days triggers mechanisms that increase the amplitudes of excitatory quantal currents. (B) Increasing activity produces effects opposite those produced by blocking activity. Increasing activity triggers mechanisms that decrease the amplitudes of excitatory quantal currents. These adjustments after activity perturbations suggest that the amplitudes of quantal currents can be homeostatically regulated. (C) Plotting the amplitudes of excitatory quantal currents as a cumulative

distribution reveals that increasing activity causes a leftward shift in amplitude suggesting that the strength of all excitatory synapses are decreased. Decreasing activity shifts the cumulative distribution of amplitudes of all excitatory currents to the right, suggesting that all amplitudes are increased after activity block. (D) Both the activity-deprived and activity-enhanced distributions collapsed onto the control (normal activity) distribution when the activity-deprived distribution was divided by a factor of 1.2 and the activity-enhanced distribution was multiplied by a factor of 2.3. This process, termed homeostatic synaptic scaling, suggests that the strength of all synapses can be increased of decreased by the same factor after activity perturbations. Scaling of the strength of synapses could provide a way for the strength of all synapses to be adjusted in a global manner while the relative differences between the strength of synapses is maintained.

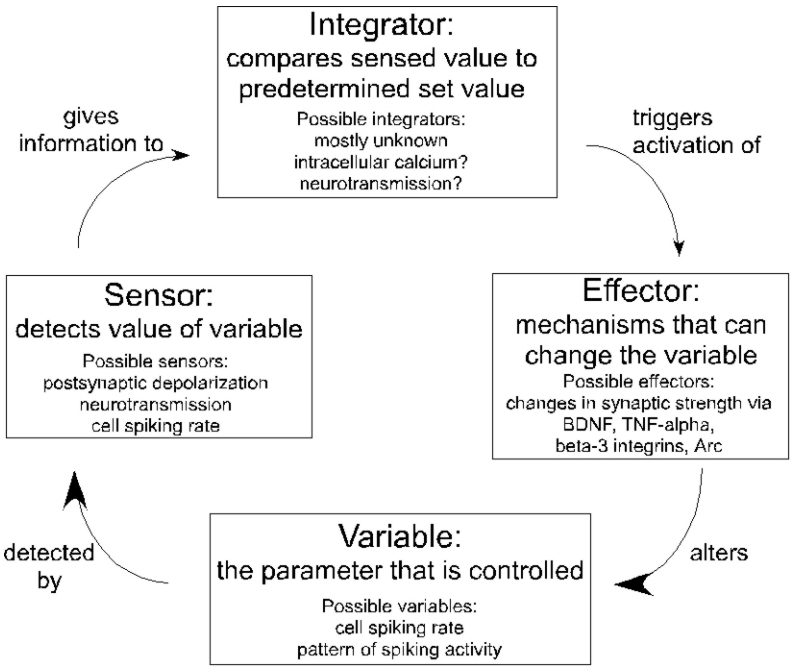

Figure 1.3. Homeostatic synaptic plasticity is likely regulated by a closed feedback loop. In this feedback loop a variable(s) is sensed by a sensor(s), which can be detected by integrator(s) that triggers an effector(s) that can recover and/or maintain the variable(s) within a predetermined set range. For example: Activity (variable) can be sensed by a reduction in $GABA_A$ receptor signaling (sensor). The change of $GABA_A$ receptor signaling could alter intracellular calcium concentrations (integrator), which could trigger a change in synaptic strength (effector). It is possible that molecules can be both sensors and integrators in this scheme. This flow chart has been modified from a similar chart in (Macleod and Zinsmaier, 2006).

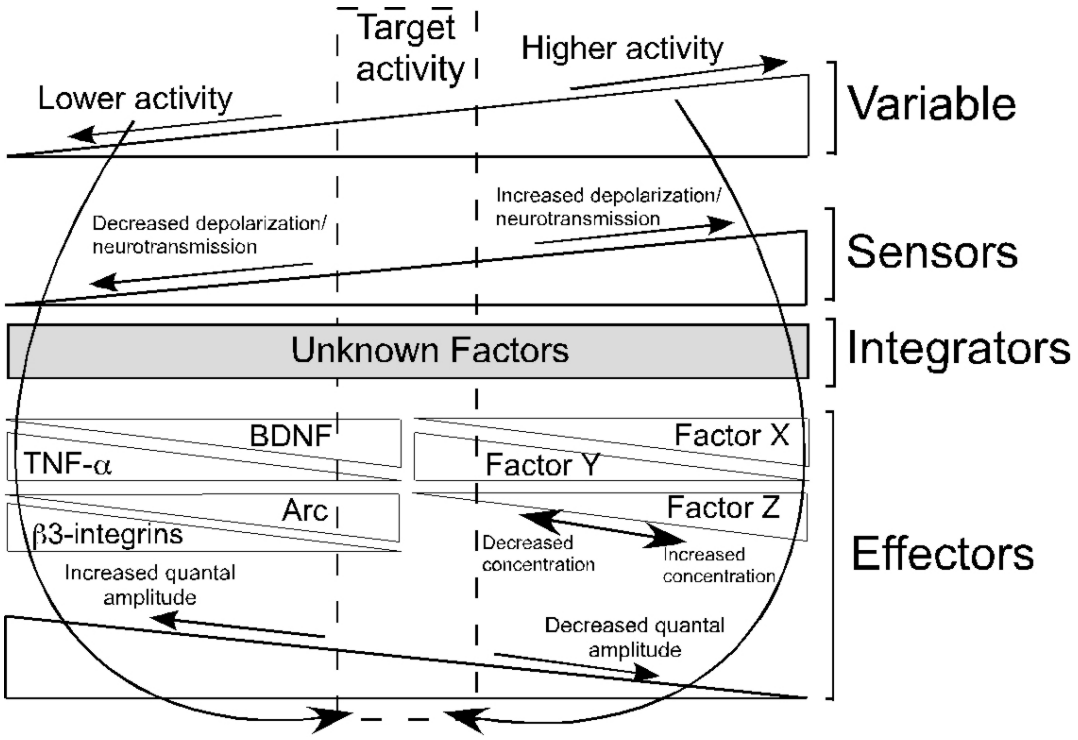

Figure 1.4. Activity dependent regulation of multiple factors could contribute to scaling of quantal amplitudes. Reductions in spiking activity can be sensed as a reduction in the average postsynaptic depolarization or as a reduction in neurotransmission. Reduction in these sensors could be monitored by unknown factors. These integrators can trigger mechanisms to increase quantal amplitude (effectors). Several possible effectors are TNF-alpha, beta-3 integrins, BDNF, and Arc. Reduced activity reduces the release of BDNF and Arc signaling while increasing TNF-alpha release and TNF-alpha dependent surface expression of beta-3 integrins. These factors, together with possible unknown factors, increase quantal amplitude, thereby, moving activity levels back towards target range. Increases in activity can trigger similar cascades, although the factors involved in recovering normal activity levels are largely unknown. Modified from (Turrigiano, 2006).

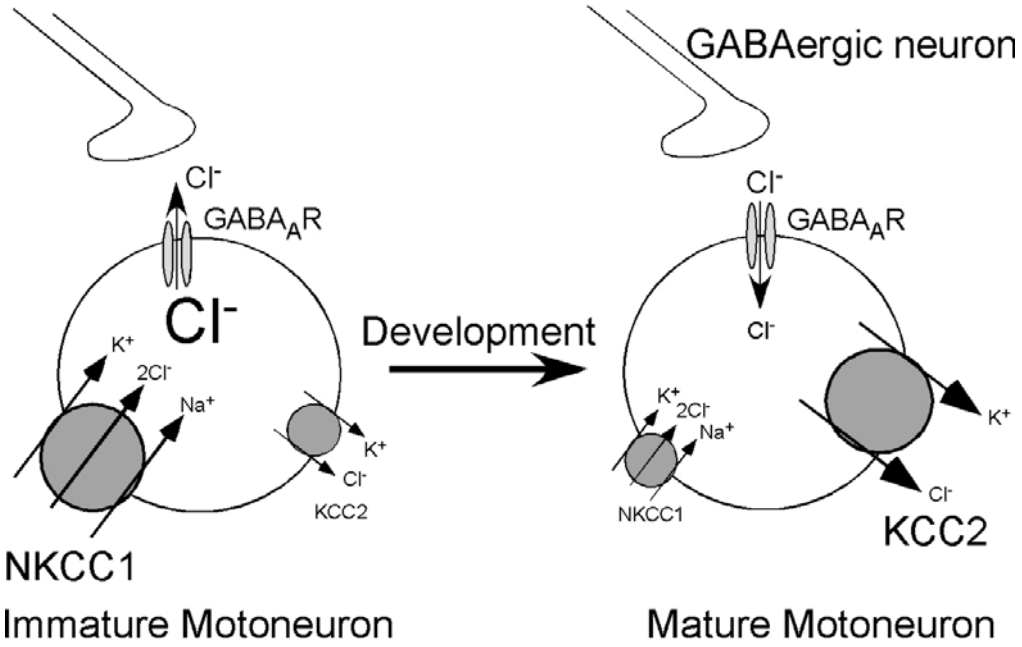

Figure 1.5. GABAergic signaling is depolarizing in early development. High intracellular chloride levels during development lead to the efflux of chloride through $GABA_A$ receptors when GABA binds producing a depolarizing postsynaptic effect. The high intracellular chloride concentration is due to an increased expression of the Cl^- accumulator NKCC1. During development NKCC1 expression is down-regulated and the expression of the chloride extruder KCC2 is up-regulated. This change in chloride pumping reduces intracellular chloride levels. Therefore, in mature neurons, GABA binding to the $GABA_A$ receptor produces the influx of chloride, which hyperpolarizes neurons.

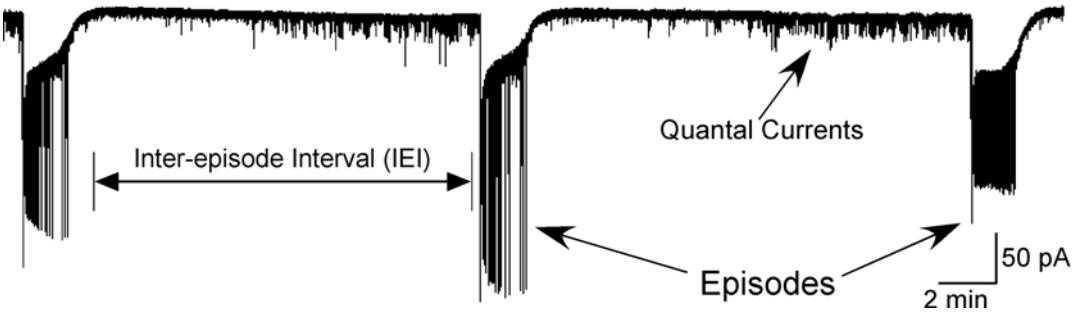

Figure 1.6. Episodes of SNA in the *in vitro* chick spinal cord. This representative trace obtained from an adductor motoneuron using whole cell voltage clamp shows that episodes of bursting are followed by periods in which few action potentials occur (the inter-episode interval). No action potentials occur in the trace shown. Also note that the amplitudes of quantal currents recorded during the inter-episode interval are smaller just after an episode and increase throughout the duration of the interval, with the largest amplitudes occurring just before the onset of the next episode. This modulatory feature is called post-episode depression of quantal currents and is due to changes in the intracellular chloride concentration.

CHAPTER 2:

$GABA_A$ TRANSMISSION IS A CRITICAL STEP IN THE PROCESS OF TRIGGERING HOMEOSTATIC INCREASES IN QUANTAL AMPLITUDE

Modified from the following reference:

Wilhelm, Jennifer C. and Wenner, Peter A. (2008). $GABA_A$ transmission is a critical step in the process of triggering homeostatic increases in quantal amplitude. Proceedings of the National Academy of Sciences, USA. 105, 11412-11417.

Abstract

When activity levels are altered over days, a network of cells is capable of recognizing this perturbation and triggering several distinct compensatory changes that should help to homeostatically recover and maintain the original activity levels. One feature commonly observed following activity blockade has been a compensatory increase in excitatory quantal amplitude. The sensing machinery that detects altered activity levels is a central focus of the field currently, but thus far has been elusive. The vast majority of studies that reduce network activity also reduce neurotransmission. In this chapter we confirm previous reports that chronic network activity blockade triggers homeostatic increases in excitatory AMPA and GABA quantal amplitude. Then, we address the possibility that reduced neurotransmission, independent of activity, can trigger increases in quantal amplitude. We block glutamatergic or $GABA_A$ transmission *in ovo* for 2 days while maintaining relatively normal network activity. We find that reducing $GABA_A$ transmission triggers compensatory increases in both GABA and AMPA quantal amplitude in embryonic spinal motoneurons, despite relatively normal activity levels. On the other hand, glutamatergic blockade does not have any affect on quantal amplitude. Therefore, GABA binding to the $GABA_A$ receptor appears to be a critical step in the sensing machinery for some types of homeostatic synaptic plasticity. These findings suggest that GABA may be serving as a proxy for activity levels when triggering homeostatic changes in synaptic strength.

Introduction

Spontaneous network activity (SNA) is a prominent feature of developing neural networks that consists of episodic bursts of activity separated by periods of quiescence (Feller, 1999; O'Donovan, 1999; Ben-Ari, 2001). SNA is produced by hyperexcitable, recurrently connected circuits in which glutamate and GABA are both excitatory early in development. In the developing spinal cord, SNA drives embryonic limb movements and is known to be important for various aspects of limb (O'Donovan et al., 1998b) and motoneuron development (Casavant et al., 2004; Hanson and Landmesser, 2004). Reducing spontaneous network activity in the embryonic chick *in vivo* leads to compensatory increases in the strength of excitatory GABAergic and AMPAergic synapses (Gonzalez-Islas and Wenner, 2006). These compensatory increases in synaptic strength have been described in several systems where they appear to act in a manner to homeostatically recover and maintain network activity levels, and are referred to as homeostatic synaptic plasticity (Rich and Wenner, 2007; Turrigiano, 2007).

Activity perturbations lead to several different forms of homeostatic synaptic plasticity including changes in quantal amplitude, probability of release, and frequency of miniature postsynaptic currents (mPSCs). In the present study we focus on compensatory changes in quantal (mPSC) amplitude. Changes in mPSC amplitude have been intensely studied in cultured networks where prolonged activity reduction results in an increase in the amplitude of excitatory mPSCs and a decrease in the amplitude of inhibitory mPSCs (O'Brien et al., 1998; Turrigiano et al., 1998; Kilman et al., 2002). Changes in mPSC amplitude

are achieved through alterations in the number of postsynaptic receptors and/or the amount of transmitter released per vesicle (Rich and Wenner, 2007; Turrigiano, 2007). However, far less is known about the machinery that senses perturbations in the level of activity, which then trigger compensatory changes in mPSC amplitude.

Several signals have been suggested as possible sensors for tracking changes in activity, including spike rate, depolarization and intracellular calcium levels (Liu et al., 1998; Marder and Prinz, 2002; Davis, 2006; Rich and Wenner, 2007; Turrigiano, 2007). One of the favored models suggests that reducing network activity produces a corresponding reduction in cellular spiking activity, thereby reducing intracellular calcium levels in a postsynaptic cell. In this model, which we will refer to as the cell activity model, the postsynaptic cell senses changes in intracellular calcium levels as a measure of altered activity and triggers compensatory changes in mPSC amplitude. However, the studies that have inspired this model not only block spiking activity, they also block or reduce neurotransmitter binding to its receptor and any associated downstream signaling cascades. Thus it remains possible that neurotransmission is a critical step in the sensing process that triggers changes in quantal amplitude. In an alternative model (the neurotransmitter model), changes in activity levels could be sensed by alterations in neurotransmitter receptor binding. In order to address such a possibility one would need to block either activity or neurotransmission, but not both. Some recent studies demonstrate that neither hyperpolarization nor reducing spike rate in the postsynaptic cell lead to compensatory changes in

mPSC amplitude as would be predicted by the cell activity model (Paradis et al., 2001; Burrone et al., 2002; Hartman et al., 2006; Pratt and Aizenman, 2007; Rich and Wenner, 2007). In those studies, the release and binding of neurotransmitters to their receptors was not altered, and thus the results are consistent with the neurotransmitter model. In order to directly test the neurotransmitter model one would like to block neurotransmission without affecting activity levels.

In the present study, we confirmed the results of our previous study (Gonzalez-Islas and Wenner, 2006) by examining the effects of activity block on synaptic strength. We extend these findings and aim to determine whether lidocaine-induced reduction in neurotransmitter triggers increases in excitatory synaptic strength. To address this question, we block GABAergic or glutamatergic neurotransmission in the developing spinal cord *in vivo*, while maintaining significant levels of SNA because of the fast compensatory nature of the highly excitable developing cord (Marchetti et al., 2005; Chub et al., 2006). We have found a particular importance of $GABA_A$ transmission in triggering compensatory changes in quantal amplitude. Our findings suggest that the $GABA_A$ receptor is part of the sensing machinery for synaptic homeostatic plasticity in the developing spinal cord.

Results

Activity block *in ovo*

Previously, we found that reducing SNA led to increases in GABAergic and AMPAergic mPSC amplitude at E10 (Gonzalez-Islas and Wenner, 2006). In order to confirm these results, we infused either saline (1ml) or the sodium channel antagonist lidocaine (35 µg/ µl at a rate of 17.5 µl/hr; assume a 50 ml egg volume) *in ovo* onto the chorioallantoic membrane of E8 embryos for 2 days (from embryonic day 8-10; E8-10). Embryonic limb movements were assessed to determine the effects of activity block on spontaneous network activity *in ovo*. Spinally generated embryonic limb movements were observed through windows in the eggshell. SNA was quantified *in ovo* by counting the total time the chick embryo moved during a 5-minute period of observation (Figure 2.1). The duration of limb movements are greatly reduced as the lidocaine concentration increases *in ovo*. The progressive reduction in limb movements is likely due to an increasing concentration of lidocaine in the egg over time.

Activity block increased SNA frequency *in vitro*

After 48 hours of lidocaine or saline treatment *in ovo*, the embryos were dissected and spinal cords were isolated with muscle nerves intact. Extracellular recordings were obtained using suction electrodes attached to muscle nerves (femorotibialis and adductor) and the ventral lateral funiculus. All recordings were performed in the absence of lidocaine. A significant decrease in the duration of the interval between episodes of SNA was found in embryos treated

with lidocaine (Figure 2.2B) suggesting that activity block decreased the threshold for the initiation of episodes. As the duration of the interval between the episodes has been shown to be correlated to the duration of the following episode (Tabak et al., 2001), we also measured the duration of the episode. We found that the duration of the episodes of SNA also was decreased (Figure 2.2C). Thus, episodes are briefer and occurring more often after lidocaine treatment. These results were consistent with those reported in Gonzalez-Islas and Wenner (Gonzalez-Islas and Wenner, 2006).

Activity block increased mPSC amplitude

To test the consequences of 48 hours of activity blockade on synaptic strength, we obtained whole cell voltage clamp recordings (holding potential -70 mV) from antidromically-identified motoneurons of embryos treated from E8 - 10 with saline or lidocaine (see methods). Recordings of mPSCs were obtained in the absence of lidocaine, TTX, or other neurotransmitter receptor antagonists unless otherwise indicated. GABA- and AMPA-mPSCs were isolated by their decay kinetics as described previously (Gonzalez-Islas and Wenner, 2006) and in the methods.

Similar to the previous findings by Gonzalez and Wenner (Gonzalez-Islas and Wenner, 2006), GABAergic mPSC amplitude was significantly increased after 48 hours of lidocaine treatment, compared to controls (39% increase; Figure 2.3, Table 2.1). We also observed that the entire distribution of GABAergic mPSCs increased by a multiplicative factor of 1.34 (Figure 2.3F), suggesting that

activity block triggered a process of synaptic scaling (Turrigiano et al., 1998). No significant differences were found in the decay or rise kinetics or in the frequency of $GABA_A$ mPSCs between saline- and lidocaine-treated embryos (Table 2.1). No significant differences in passive membrane properties were found between the lidocaine- or saline-treated groups (Table 2.2). These results are consistent with the cell activity model, in which activity block triggers changes in mPSC amplitude.

We also confirmed that the average amplitude of AMPAergic mPSCs from lidocaine-treated motoneurons was significantly increased compared to controls [34% increase; Table 2.1, Figure 2.3; (Gonzalez-Islas and Wenner, 2006)]. We did not find any significant differences in the rise or decay kinetics of AMPA-mPSCs between the two groups (Table 2.1).

Unlike the previous findings (Gonzalez-Islas and Wenner, 2006), we did not observe a change in the frequency of AMPAergic mPSCs (Table 2.1). The frequency of AMPA-mPSCs from control embryos in this study (0.8 ± 0.1 Hz) is higher compared to the frequency of AMPA-mPSCs from control embryos reported in the previous study (0.2 ± 0.1 Hz). In the previous study (Gonzalez-Islas and Wenner, 2006), motoneurons from lidocaine-treated embryos had a significant increase in the frequency of AMPA-mPSCs to 0.7 ± 0.1 Hz ($p < 0.0001$). The reasons behind this difference in measured frequency are unclear. A difference in dissection technique (stage of chick, temperature of dissection, etc.), in the definitions used to define an event, or in the sampling rate could have

caused an increase in the number of fast events to be counted in the current study.

We found a significant rightward shift in the cumulative distribution of AMPA-mPSC amplitudes for lidocaine-treated motoneurons, suggesting that all amplitudes were increased after lidocaine treatment. When the entire distribution of lidocaine-treated AMPA-mPSC amplitudes was divided by a factor of 1.5, the distribution nearly collapsed onto the control distribution ($p < 0.06$; Figure 2.3G). The previous study did not find scaling of the AMPA-mPSC cumulative distribution after lidocaine treatment (Gonzalez-Islas and Wenner, 2006), possibly due to the reported increase in frequency. An increase in frequency could occlude a trend towards scaling if lidocaine treatment revealed a new population of functional synapses that are not affected by activity block (Ibata et al., 2008). The emergence of new synapses that did not scale could bias the distribution of synapses that did scale, thereby obscuring the researchers ability to measure the scaling effect. On the other hand, new synapses could be less strong than existing synapses; therefore, even if all of the synapses were scaled by the same factor, the new weaker synapses would cause the distribution to appear not to scale because there would be an increase in smaller amplitude currents. It is also possible that activity blockade induces a homeostatic increase in AMPA-mPSC amplitudes that do not increase all glutamatergic synapses equally. Future experiments examining the mechanisms involved in regulating the increase in mPSC amplitudes after activity block are necessary to further

elucidate this issue (see future directions for further discussion of these experiments).

The acute addition of TTX (1 μM) to the isolated spinal cord to block action potentials did not alter AMPA- or GABA-mPSC amplitudes or frequencies of control- (see methods) or lidocaine-treated mPSCs (Gonzalez-Islas and Wenner, 2006). This suggests that all of the recorded currents were action potential independent mPSCs. To determine if increases in mPSC amplitude in lidocaine-treated embryos is due to the emergence of a new population of mPSCs with a different pharmacology, we isolated the mPSCs by acute application of bicuculline or CNQX. Bath application of bicuculline (20 μM) reduced the frequency of slow mPSCs to 4.4% suggesting that lidocaine treatment did not reveal a new population of slow mPSCs that are not GABAergic. Bath application of CNQX (20 μM) reduced fast mPSCs to 4.8% of pre-drug levels. The addition of both bicuculline and CNQX together abolished all mPSCs, suggesting that lidocaine-treatment did not reveal any new populations of mPSCs with a different pharmacology (Gonzalez-Islas and Wenner, 2006).

Together, these data support the cell activity model because 48 hours of activity blockade triggers increases in the amplitudes of GABA- and AMPA-mPSCs. However, lidocaine blockade reduces the spike-dependent release of AMPA and GABA. Thus, we cannot rule out the possibility that the reduction in neurotransmission triggered the increases in synaptic strength.

Neurotransmitter blockade *in ovo*

In order to test the possibility that reduced ionotropic neurotransmitter signaling triggered compensatory changes in quantal amplitude, we injected various neurotransmitter antagonists *in ovo*. We first determined the concentration of receptor antagonists necessary to significantly reduce neurotransmission for 48 hours *in ovo*. Embryonic limb movements, counted during a 5-minute observation period, were assessed to determine the effects of neurotransmitter receptor block on spontaneous network activity *in ovo* (Figure 2.4A). We also monitored the number of bouts of movement (Figure 2.4B), and the duration of each bout (Figure 2.4C).

To determine the effective concentrations of receptor antagonists needed to significantly block activity *in ovo*, a cocktail of ionotropic receptor antagonists was infused onto the chorioallantoic membrane of the developing chick, including a $GABA_A$ receptor antagonist (100 μM bicuculline, 50 μM - 100 μM picrotoxin, 10 μM gabazine, assuming a 50 ml egg volume), a glycine receptor antagonist (5 - 7 μM strychnine), an AMPA/kainate (referred to as AMPA from this point) receptor antagonist (20 μM CNQX), and an NMDA receptor antagonist (100 μM APV), The injection of the cocktail of these 4 antagonists at E8 resulted in a near cessation of all limb movements from embryonic day 8 to 10 (stages 33 – 36; Figure 2.4 - red).

The exclusion of any one of these drugs from the cocktail reduced limb movements but did not abolish them for the entire 2 days (not shown). The injection of a single $GABA_A$ receptor antagonist or AMPA receptor antagonist, or

a combination of AMPA and NMDA antagonists caused a temporary reduction in limb movements that recovered to control levels within hours (Figure 2.4). Doubling the concentration of any drug or the addition of a second bolus at E9 produced no additional effect on limb movements (not shown). This suggests that the drugs are still bound to their receptors, but that the spinal networks controlling limb movements have compensated for the loss of transmission (Chub and O'Donovan, 1998). These experiments demonstrate that $GABA_A$ or AMPA/NMDA receptor activation can be significantly reduced *in ovo* from E8-10, while levels of SNA remain largely intact, apart from a transient reduction following drug administration.

Neurotransmitter block does not alter SNA frequency *in vitro*

Following two days of neurotransmitter blockade *in ovo*, we isolated the embryonic spinals cords. Extracellular muscle nerve recordings were performed in the absence of receptor antagonists to assess the frequency of SNA *in vitro*. Chronic blockade of $GABA_A$ receptors or glutamatergic receptors did not significantly change the duration of the episodes (Figure 2.5A) or the duration of the interval between episodes (Figure 2.5B). This lack of increase in the frequency of episodes of SNA *in vitro* suggests that neurotransmitter receptor block and activity block do not trigger completely overlapping mechanisms.

No change in mPSCs following AMPA/NMDA receptor block

To test the consequences of blocking ionotropic glutamatergic receptor signaling, we obtained whole cell voltage clamp recordings (holding potential -70 mV) from antidromically identified motoneurons of embryos treated from E8 - 10 with saline (1 ml), the AMPA receptor antagonist CNQX (20 μM) alone, or CNQX together with the NMDA receptor antagonist APV (100 μM). Recordings of mPSCs were obtained as described above.

Chronic CNQX treatment did not result in a change in the amplitude of AMPA- or $GABA_A$-mPSCs (Figure 2.6, Table 2.1). Similarly, the average amplitudes of AMPA- or $GABA_A$-mPSCs were not significantly altered by chronic AMPA and NMDA receptor blockade (Figure 2.6, Table 2.1). Further, average mPSC frequency (Table 2.1), mPSC rise and decay kinetics (Table 2.1) and cellular passive membrane properties (Table 2.2) were unaltered following glutamate receptor blockade from E8-10. We did observe a reduction in the variability of mPSC amplitude when embryos were treated with the glutamate receptor antagonist (Figure 2.6). Although we do not know what normally produces this variability it has been suggested that networks maintain a level or pattern of activity by using different combinations of distinct parameters, each parameter exhibiting considerable variability (Prinz et al., 2004; Marder et al., 2007). If a particular conductance (synaptic receptor/potassium channel) or the probability of release is influenced by antagonist treatment, then it may be that the remaining parameters have a more restricted parameter space and therefore exhibit less variability.

These findings suggest that reducing glutamatergic transmission did not trigger compensatory changes in quantal amplitude observed following activity blockade. This finding is consistent with the cell activity model, but also the neurotransmitter model, assuming GABA is the key neurotransmitter, which was intact in these experiments. $GABA_A$ signaling has been shown to be particularly important for several aspects of early development (Ben-Ari et al., 2007). Next we considered the possibility that reducing $GABA_A$ transmission for 2 days would trigger increases in quantal amplitude.

$GABA_A$ receptor block increased mPSC amplitude

To test the consequences of blocking $GABA_A$ receptor signaling, we obtained whole cell recordings from motoneurons as described above, but from embryos treated with a $GABA_A$ receptor antagonist (bicuculline) from E8-10. GABAergic mPSC amplitude was significantly greater in embryos where $GABA_A$ receptors were blocked for 2 days, than in controls (108% increase; Table 2.1, Figure 2.7). We found that full recovery of embryonic movements did not occur until E10 following bicuculline treatment at E8 (Figure 2.4). This may occur because of nonspecific effects of bicuculline (Pflieger et al., 2002) or because bicuculline acts at both synaptic and extrasynaptic $GABA_A$ receptors. Therefore, we also treated embryos with either picrotoxin or gabazine, which is thought to be more specific for synaptic $GABA_A$ receptors (Bai et al., 2001). Surprisingly, we were unable to determine a concentration of picrotoxin that would sufficiently block $GABA_A$ receptors without killing the embryos. However, we were able to

determine an effective concentration of gabazine without altering embryonic health, and found that after a bolus injection of gabazine embryonic movements recovered more quickly than after bicuculline treatment (Figure 2.4). GABAergic mPSC amplitudes from gabazine-treated embryos were again significantly greater than controls (82% increase), but were not different from embryos treated with bicuculline (Table 2.1, Figure 2.7). We also observed that the entire distribution of GABAergic mPSCs increased by a multiplicative factor (Figure 2.7F), or in other words the distribution scaled (Turrigiano et al., 1998). No significant differences were found in the frequency of $GABA_A$ mPSCs for either treatment condition (Table 2.1). Decay and rise times for recorded GABA-mPSC waveforms were no different in control and $GABA_A$-blocked embryos (Table 2.1). Further, no significant differences were found in passive membrane properties between the groups (Table 2.2). These data suggest that blocking $GABA_A$ receptor transmission leads to an increase in the amplitude of GABAergic mPSCs.

AMPA mPSCs from $GABA_A$ receptor-blocked embryos were kinetically isolated from the same set of motoneurons described above. Chronic $GABA_A$ receptor antagonist-treatment significantly increased the amplitude of AMPA mPSCs compared to controls, even though AMPA receptors were not blocked (63% increase with gabazine, 68% increase with bicuculline; Table 2.1, Figure 2.7). Again, the entire distribution of AMPAergic mPSCs appeared to scale (Figure 2.7G). The AMPA-mPSC rise times, decay times, frequency, passive

membrane properties were not significantly altered by either bicuculline or gabazine treatment (Tables 2.1 and 2.2).

To determine if increases in mPSC amplitude in embryos treated with gabazine from E8-10 were due to a new population of mPSCs with a different pharmacology, we isolated the mPSCs by acute bath application of CNQX and/or gabazine. We found that addition of CNQX (20 μM) reduced the mPSC population with fast decay kinetics to only 6.3% of that before the antagonist. Similarly, addition of gabazine (10 μM) reduced the mPSC population with slow decay kinetics to only 4.1% of that before the antagonist. When both CNQX and gabazine were added to the bath, mPSCs were virtually abolished (n = 3). These results suggest that chronic $GABA_A$ receptor blockade did not reveal a new population of mPSCs with a different pharmacology. Additionally, acute application of TTX did not significantly change the amplitude (19.6 ± 0.2 pA; then after TTX, 18.6 ± 0.7 pA) or frequency (0.79 ± 0.22 Hz; then after TTX, 0.83 ± 0.19 Hz) of mPSCs (n = 3). Results with antagonists or TTX in gabazine-treated embryos are similar to those observed in control embryos (see methods).

12 hour $GABA_A$ block did not induce changes in mPSC amplitude.

Embryonic movements recover in either $GABA_A$ or glutamatergic block within 12 hours. One of the hallmarks of homeostatic changes in mPSC amplitude has been that it requires approximately a day of altered activity to develop. Therefore, we believed that the recovery of embryonic movements occurred through fast transiently existing changes in network excitability that

have been described for the isolated spinal cord *in vitro* (Chub and O'Donovan, 1998). Regardless, we wanted to test the possibility that changes in quantal amplitude in the embryonic spinal cord might occur faster than in other systems and contribute to the recovery of embryonic movements. In order to assess the time course of the increase in mPSC amplitude, and determine if it could contribute to the recovery of embryonic movements *in ovo*, we injected gabazine *in ovo* at E9.5 and assayed quantal amplitude 12 hours later at E10. Similar to the findings described above, *in ovo* limb movements were dramatically reduced and then recovered to near control levels by 12 hours of gabazine treatment (Figure 2.8A). Whole-cell voltage clamp recordings obtained from motoneurons of embryos treated for only 12 hours with gabazine demonstrated no significant difference in the amplitude of AMPA or $GABA_A$ mPSCs (Figure 2.8B-C, Table 2.1). Neither the frequency of GABA-mPSCs nor AMPA-mPSCs was significantly different between control and 12 hours of $GABA_A$ receptor blockade (Table 2.1). The results suggest that $GABA_A$ receptor block must be in place for greater than 12 hours to exhibit changes in mPSC amplitude that are observed in embryos treated from E8-10. Further, the findings suggest that the initial recovery of embryonic movements 12 hours after injection of gabazine occurs without the contribution of increases in mPSC amplitude. Finally, in embryos treated from E8-10 mPSC, amplitude must increase during the period when SNA levels were relatively normal.

Although 48 hours of glutamate receptor block did not trigger changes in the amplitude of GABAergic or AMPAergic mPSCs, we tested whether 12 hours

of CNQX or 12 hours of CNQX + APV produced changes in AMPA- and GABA-mPSCs. Unsurprisingly, 12 hours of glutamate receptor block did not alter the amplitude or frequency of AMPA- or GABA-mPSCs (Table 2.1). Together, these results suggest a particular importance for $GABA_A$ transmission in triggering homeostatic changes in GABAergic and AMPAergic mPSC amplitudes.

Activity block, but not $GABA_A$ receptor block, accelerates the rate of recovery of GABA-mPSC amplitude from post-episode depression during the inter-episode interval.

During the inter-episode interval, GABA-mPSC amplitude undergoes a fast, transient reduction that recovers before the next episode (Fedirchuk et al., 1999; Chub and O'Donovan, 2001). This recovery may contribute to the generation of the next episode (Tabak et al., 2000; Tabak et al., 2001; Marchetti et al., 2005). Separating mPSCs into 2-minute bins allows us to analyze the recovery of mPSC amplitudes to pre-episode levels (Figure 2.9A). The average mPSC amplitude from each bin is compared sequentially to determine the rate of recovery. All bins during an inter-episode interval are normalized to the last 2-minute bin just before the previous episode, such that the value in the last 2 minutes prior to the episode becomes 100%.

The amplitude of GABA-mPSCs in the first 2-minute bin after the episode were reduced to approximately 60% (lidocaine) or 68% (control) of GABA-mPSC amplitude in the last 2-minute bin prior to the episode (Figure 2.9B). This finding suggests that lidocaine treatment does not alter the degree of depression of GABA-mPSC amplitudes after an episode of SNA. GABA-mPSC amplitudes

fully recover before the next episode in both lidocaine- and saline-treated embryos. The amplitudes of GABA-mPSCs from both groups become progressively larger during the inter-episode interval until the pre-episode amplitudes are completely recovered (Figure 2.9B). However, we found that the rate at which GABA-mPSC amplitudes recover from the post-episode depression is accelerated in activity-reduced, lidocaine-treated preparations (Figure 2.9B). AMPA-mPSC amplitudes do not appear to modulate during the inter-episode interval regardless of saline or lidocaine treatment (Figure 2.9C). These findings were also demonstrated in our previous study (Gonzalez-Islas and Wenner, 2006). This acceleration of recovery of GABA-mPSCs from post-episode depression makes sense, as the interval between episodes is significantly reduced in lidocaine-treated embryos. This increase in recovery may partly mediate the increase in the frequency of episodes of SNA found in activity-reduced preparations.

To test the possibility that the rate of recovery of the amplitudes of GABA-mPSCs from post-episode depression is accelerated after $GABA_A$ receptor blockade, we analyzed AMPA- and GABA-mPSC amplitudes sequentially during the inter-episode interval as described above. Chronic blockade of $GABA_A$ receptors produced a depression in GABA-mPSC amplitudes (60% for gabazine, 65% for bicuculline) similar to the depression seen in controls after the episode. Unlike after lidocaine treatment, the recovery of GABA-mPSC amplitudes from post-episode depression is not altered by bicuculline or gabazine treatment (Figure 2.9D). Consistent with our findings in control and lidocaine-treated

embryos, AMPA-mPSC amplitudes from bicuculline- and gabazine-treated embryos did not appear to modulate (Figure 2.9E). Our finding that GABA-mPSC amplitudes did not recover faster after chronic $GABA_A$ receptor block may not be unexpected if the rate of recovery from post-episode depression regulates the frequency of episodes of SNA. Neither gabazine nor bicuculline treatment alters the frequency of episodes of SNA *in vitro*; thus, we might not predict a change in the rate of recovery. These results suggest that while blocking activity or receptor transmission may have similar effects on many aspects of synaptic strength, there are differences as well.

Discussion

In this study we aimed to determine if neurotransmission was a critical part of the sensing machinery that triggers homeostatic changes in mPSC amplitude after perturbations to network activity levels. In a previous study we demonstrated that reducing SNA *in ovo* produced an increase in the amplitude of both GABA- and AMPA-mPSCs (Gonzalez-Islas and Wenner, 2006). These increases could be triggered by reductions in cellular activity (e.g. spike rate) leading to a reduction in intracellular calcium levels, which could be sensed by the cell and trigger changes in the amplitude of mPSCs in that cell [cell activity model; (Thiagarajan et al., 2002; Thiagarajan et al., 2005; Ibata et al., 2008)]. Alternatively, the reduction in SNA could cause a reduction in the release of neurotransmitter, which would cause a reduction in binding of neurotransmitter to its receptor and this would trigger compensatory changes in quantal amplitude

(neurotransmitter model). In this study we show evidence *in vivo* supporting the neurotransmitter model rather than the cell activity model. *In ovo* block of $GABA_A$ receptors triggered a strong increase in the amplitude of both $GABA_A$ and AMPA mPSCs, despite the fact that SNA was maintained, as embryonic movements were largely intact in gabazine-treated embryos. This places GABAergic neurotransmission as a critical step in the homeostatic process. Because $GABA_A$ receptors are blocked throughout the network, the key site of neurotransmission block that triggers quantal amplitude changes could be at the postsynaptic cell, the presynaptic cell, and/or the resident glia. These findings support the idea that when SNA is reduced, $GABA_A$ transmission is also reduced and this triggers the compensatory increase in quantal amplitude. Therefore, levels of released GABA would be monitored as a proxy for activity.

Transiently reducing SNA levels does not increase mPSC amplitudes.

Embryonic movements are likely to be a reliable indicator of spinal SNA as electromyographic recordings *in ovo* during embryonic movements are remarkably similar to motoneuron recordings during *in vitro* SNA (Provine, 1972; Provine and Seshan, 1973; Landmesser and O'Donovan, 1984; O'Donovan, 1989). It is also likely that SNA that recovers after antagonist injections is similar to SNA before the antagonists were added. *In vitro* muscle nerve recordings from the isolated spinal cord show that SNA that has recovered following block of glutamatergic or GABAergic/glycinergic transmission was very similar to SNA before antagonists were added (Chub and O'Donovan, 1998). Further, we did

not observe differences in several features of the *in ovo* embryonic movements in treated and control embryos, including total movement duration, bout duration, bout frequency, or recovery of the movements. We remain open to the possibility that subtle distinctions between the embryonic movements and SNA may exist. However, it is unlikely that increases in quantal amplitude are triggered by low activity levels in $GABA_A$-blocked embryos since they have larger increases in mPSC amplitude than activity-blocked lidocaine-treated embryos (Gonzalez-Islas and Wenner, 2006). For this reason it is also highly unlikely that a transient reduction in SNA, following antagonist injection, could account for the observed increase in quantal amplitude. Blocking $GABA_A$ receptors for 12 hours, or blocking glutamate receptors for 48 hours produced the same transient reduction and recovery in embryonic movements as 48 hour $GABA_A$-block, but failed to produce the changes in mPSC amplitude observed following 48 hour GABA-block. These findings suggest that lowered GABAergic neurotransmission triggers compensatory changes in quantal amplitude. The results are consistent with the idea that GABAergic transmission is partly reduced in lidocaine-treated embryos (action potential dependent release), but is more completely reduced in the presence of $GABA_A$ antagonists. Taken together the findings suggest that chronic blockade of $GABA_A$ receptors, rather than a reduction in activity, triggers changes in mPSC amplitude, thereby supporting the neurotransmitter hypothesis.

Changes to mPSC amplitude following neurotransmitter block.

At E10, the two dominant spinal motoneuron inputs are GABAergic and glutamatergic (Gonzalez-Islas and Wenner, 2006). Blocking AMPA and NMDA receptors failed to trigger changes in the amplitude of mPSCs. Previous studies in cultured networks also have found that NMDA receptor block did not trigger compensatory changes in mPSC amplitude (O'Brien et al., 1998; Turrigiano et al., 1998). Our findings suggest a special role for GABAergic transmission in triggering the compensatory changes in mPSC amplitude. This places GABA and its receptor high in the hierarchical cascade that leads to downstream changes in both $GABA_A$ and AMPA mPSC amplitude. GABA may be capable of triggering changes in quantal amplitude in early development because it is depolarizing and GABAergic synapses may be more fully developed than the AMPAergic system at this stage (Chen et al., 1995; Ben-Ari et al., 2004). It is now well appreciated that GABA signaling is involved in directing multiple developmental processes, including: cell proliferation, migration, and differentiation; establishment of synaptic connections and their refinement; and potentially in the depolarizing to hyperpolarizing conversion of the $GABA_A$ response itself (Owens and Kriegstein, 2002; Kandler and Gillespie, 2005; Akerman and Cline, 2007; Ben-Ari et al., 2007).

Is it possible that reduced neurotransmission also triggers compensatory changes in mPSC amplitude in other systems where homeostatic synaptic plasticity has been described? In most studies where cultured network activity levels are perturbed, so too are released transmitters; thus, it is possible that

altered neurotransmission triggers changes in mPSC amplitude in these studies as well (Lissin et al., 1998; O'Brien et al., 1998; Turrigiano and Nelson, 1998). In cultured networks, reducing $GABA_A$ transmission triggers a reduction in the amplitude of excitatory mPSC amplitude, which makes functional sense because in these studies GABA is inhibitory and blocking it increases spiking activity (Lissin et al., 1998; O'Brien et al., 1998; Turrigiano et al., 1998). This is opposite to our finding of increased quantal amplitude following $GABA_A$-block, though, in embryonic spinal neurons GABA is depolarizing. As a result, GABA may be able to activate downstream signaling cascades early in development because $GABA_A$ transmission can lead to calcium entry. Once $GABA_A$ transmission is no longer depolarizing it may not be able to trigger the same downstream cascades, and the role of triggering compensatory quantal changes may be transferred to AMPAergic transmission. In four separate studies, (*in vitro and in vivo*) activity was reduced in only the postsynaptic cell by expression of a potassium channel, while neurotransmitter release by the cell's inputs remained intact and presumably unaltered (Paradis et al., 2001; Burrone et al., 2002; Hartman et al., 2006; Pratt and Aizenman, 2007). While some of these studies reported a compensatory change in probability of release, none of them observed an increase in quantal amplitude, despite the reduction of spiking activity. These studies are therefore consistent with the neurotransmitter model. Further, in a recent study of homeostatic synaptic plasticity in hippocampal cultured networks, Stellwagen and Malenka (Stellwagen and Malenka, 2006) show that compensatory increases in AMPAergic mPSC amplitude are triggered by glia

that may sense low glutamate levels in the culture media following reduced activity. Thus, our findings of reduced neurotransmission triggering homeostatic changes in quantal amplitude may be a generally applicable finding for homeostatic synaptic plasticity in conditions of lowered activity. On the other hand, a recent study reports that spiking activity is important in triggering a fast form of AMPAergic quantal scaling (≤ 4 hours) that was dependent on the glial conditions of the culture system (Ibata et al., 2008). It is known that the mechanisms of homeostatic synaptic plasticity following reductions in activity are distinct from those following increased activity (Turrigiano and Nelson, 2004), and one study suggests that depolarization rather than transmission may be important in triggering compensatory changes in quantal amplitude in response to increased network activity (Leslie et al., 2001).

Activity and neurotransmission may regulate different aspects of homeostasis.

Our findings suggest that activity block and $GABA_A$ receptor block both produce increases in the amplitudes of excitatory quantal currents. However, we found an increase in the frequency of SNA *in vitro* only after activity block or when the majority of neurotransmission was blocked using a cocktail of receptor antagonists. This finding may appear counterintuitive because one would expect that increasing synaptic strength would increase the excitability of the network, thereby increasing the frequency of episodes of SNA. In fact, Gonzalez-Islas and Wenner (Gonzalez-Islas and Wenner, 2006) demonstrated a relationship between synaptic strength and SNA frequency. Reducing synaptic strength by

applying a half-maximal concentration of neurotransmitter receptor antagonists did reduce the frequency of episodes of SNA *in vitro*. Although this suggests that synaptic strength is important in the generation of episodes of SNA, other mechanisms are likely to also be involved. Currently the mechanisms that regulate the temporal pattern of SNA are not fully understood (O'Donovan et al., 1998a). The speed of recovery from post-episode depression is likely one important mechanism that is involved in the generation of episodes (Tabak et al., 2000; Tabak et al., 2001; Marchetti et al., 2005). We found that the recovery from post-episode depression is accelerated after activity block but not after $GABA_A$ receptor block. This difference in recovery could account for the difference in expression of SNA frequency. The reasons why $GABA_A$ receptor block does not accelerate the recovery from post-episode depression in a manner similar to activity block are unclear. In addition to the rate of recovery from post-episode depression, multiple other mechanisms are probably involved in the generation of SNA. It will be important to determine the mechanisms responsible for the generation of episodes of SNA to better appreciate the differences in the frequency of episodes of SNA between lidocaine-and gabazine-treated embryos.

Recovery of SNA in ovo is not due to changes in quantal amplitude.

Within 12 hours of the injection of gabazine or CNQX + APV, embryonic movements recover to control levels. CNQX + APV treatment does not produce changes in quantal amplitude, and $GABA_A$-block takes more than 12 hours to

increase quantal amplitude. Therefore, the recovery of embryonic movements occurs in the absence of changes in quantal amplitude, and as a result must involve other mechanisms. It is likely that several mechanisms are recruited to recover and maintain activity levels, and that the different mechanisms may have different time courses (Davis, 2006). Following glutamatergic blockade activity recovers in part because of a fast loading of intracellular chloride, which strengthens the unblocked GABAergic currents (Chub and O'Donovan, 1998, 2001). Following GABAergic block there may be fast changes in probability of release (Frank et al., 2006) and changes in cellular excitability (Desai et al., 1999c; Marder and Goaillard, 2006). We have found that following $GABA_A$ block *in ovo*, there are fast changes (≤ 12 hrs) in cellular excitability that begin to dissipate toward control levels after 48 hours of $GABA_A$-block (see chapter 3). Therefore it is possible that SNA recovers following gabazine application through fast changes in cellular excitability, and that compensatory changes in mPSC amplitude take longer to be expressed. Similar findings have been reported in activity-blocked cultured networks where changes in current densities of different voltage gated channels increased the excitability of the neurons (Desai et al., 1999c; Marder and Goaillard, 2006). The precise details of the compensatory responses underlying the homeostatic recovery of activity levels is not well understood, in part because studies have not directly compared the time course of activity recovery and changes in quantal amplitude. Further, studies tend to focus on one aspect of homeostasis, synaptic or intrinsic excitability. It will be

important to consider these compensatory mechanisms together to gain a more complete understanding of the process of homeostatic recovery.

Concluding remarks

Taken together, our findings suggest that a reduction in $GABA_A$ receptor activation triggers compensatory changes in quantal amplitude in the developing spinal cord. The results support the hypothesis that lowered activity is sensed through reduced release of GABA, via $GABA_A$ receptors. This would make $GABA_A$ receptors a key determinant in the sensing machinery that triggers compensatory changes to maintain network activity in the developing nervous system, and have implications for the role of neurotransmission in homeostatic synaptic plasticity in general.

Experimental Procedures

Dissection.

White Leghorn chicken embryos (Hy-Line Hatcheries, Mansfield, GA) were dissected as described previously (Wenner and O'Donovan, 2001). Briefly, spinal cords from stage 36 (E10) embryos were isolated with intact muscle nerves arising from the crural plexus. The preparation was maintained in a re-circulating bath of oxygenated Tyrode's solution and the dissection was performed at 15º C and left overnight at 17º C. Recordings were performed in oxygenated Tyrode's solution with 5 mM KCl. Muscle nerves (femorotibialis external/medial, adductor) were drawn into suction electrodes for stimulation and

recording. The dissection and recordings were performed in the absence of drugs unless otherwise stated.

Lidocaine infusion.

At stage 33-34 [E8, (Hamburger and Hamilton, 1951)], a window in the shell was opened to allow monitoring of chicken embryo limb movements and drug application. Lidocaine hydrochloride (Sigma-Aldrich) aqueous solution (35 µg/µl plus 10 mM HEPES; pH 7.2-7.3) was continuously perfused onto the chorioallantoic membrane of the chick embryos at a rate of 13.5 µl/hr with an infusion pump. This infusion technique has been shown previously to reduce SNA for 48 hours without altering chick development or viability (Gonzalez-Islas and Wenner, 2006).

Pharmacological blockade of synaptic transmission.

An aqueous solution of (-)-bicuculline methiodide (100 µM; Tocris Cookson), gabazine (or SR 95531 hydrobromide; 10 µM; Tocris Cookson), strychnine hydrochloride (7 µM; Sigma-Aldrich), 6-Cyano-7-nitroquinoxaline-2,3-dione disodium (CNQX, 20 µM; Tocris Cookson), picrotoxin (50-100µM; Tocris Cookson) DL-2-Amino-5-phosphonopentanoic acid (APV; 100 µM; Sigma-Aldrich), or some combination of these antagonists was injected at E8 as a bolus onto the chorioallantoic membrane of the chick embryo.

Whole cell electrophysiology: voltage clamp.

Whole cell voltage clamp recordings of mPSCs were obtained from antidromically identified femorotibialis or adductor motoneurons as previously described [(Gonzalez-Islas and Wenner, 2006); Figure 2.10). Muscle nerve recordings were obtained from suction electrodes connected to high-gain differential amplifiers (A-M systems). Whole cell currents were acquired using an AxoClamp 2B amplifier controlled by pClamp 10.1 software (Axon Instruments) running on a laptop computer (Apple). Only cells with a stable resting membrane potential were used for analysis (MiniAnalysis, Synaptosoft, Decatur, GA).

Extracellular and intracellular solutions.

All recordings were performed in oxygenated (5% $C0_2$/95% O_2) saline solution at 27ºC. For all the external solution, the osmolarity was 305-315 mOsm. The osmolarity for the intracellular solution was 289-291 mOsm. All solutions had a pH of 7.2-7.3.

The external solution for recordings was (in mM): NaCl (139), KCl (5), $CaCl_2$ (3), $MgCl_2$ (1), $NaHCO_3$ (17), and D-glucose (12). The intracellular solution was (in mM): NaCl (10), KCl (36), K-gluconate (94), CaCl2 (0.1), $MgCl_2$ (1), HEPES (10), EGTA (1.1), Na_2-ATP (1), and Mg-GTP (0.1).

Identification and characterization of spontaneous postsynaptic currents.

Synaptic strength was assessed by measuring the amplitude and the frequency of spontaneous postsynaptic currents (sPSCs). Previous studies

using tetrodotoxin (TTX) have demonstrated the majority of sPSCs recorded are action potential independent mPSCs (Chub and O'Donovan, 2001; Gonzalez-Islas and Wenner, 2006). We found that acute TTX treatment did not alter the measured amplitudes or frequencies of AMPA- or GABA-mPSCs (Table 2.3). Therefore, experiments were performed without TTX (except where mentioned) to minimize potential changes in mPSC amplitude due to long-term TTX application and are referred to as mPSCs.

Previous reports have suggested that motoneurons in the E10 chick embryo primarily receive GABAergic and glutamatergic mPSCs (Chub and O'Donovan, 2001; Gonzalez-Islas and Wenner, 2006). To establish which populations of neurotransmitters contributed to the mPSCs recorded, GABAergic and glutamatergic antagonists were acutely bath applied (Figure 2.11). As all mPSCs are recorded at -70 mV, we do not observe NMDA-mediated mPSCs. We found that mPSCs were sensitive to either the $GABA_A$ receptor antagonist gabazine (20 μM) or the AMPA/kainate receptor antagonist CNQX (20 μM). Virtually no mPSCs remained after the addition of both antagonists (Figure 2.11). These findings support the idea that mPSCs in femorotibialis and adductor motoneurons at E10 are composed of only two populations, AMPA-mPSCs and GABA-mPSCs.

Previous studies suggested that AMPA- and GABA-mPSCs could be isolated kinetically (Galante et al., 2000; Weiner et al., 2005; Gonzalez-Islas and Wenner, 2006). Using receptor antagonists, the average decay time (τ) was determined for AMPA- and $GABA_A$-mPSCs. Consistent with the previous

studies, we found that in the chick embryo slow decay mPSCs are largely GABA mediated; whereas, fast decay mPSCs are largely AMPA mediated. We also determined that the average mPSC amplitude and frequency are similar for mPSCs isolated by decay kinetics or isolated by receptor antagonists (Figure 2.12). We found that only 6.6% of mPSCs with decay constants above 23 ms (presumptive GABAergic) are AMPAergic (based on pharmacologically isolated mPSCs in those cells, n = 4). Only 7.5% of mPSCs with decay constants below 21 ms (presumptive AMPAergic) are GABAergic (n = 3). Therefore, we kinetically separated contributions from GABAergic- ($\tau \geq 23$ ms) and AMPAergic-mPSCs ($\tau \leq 21$ ms) without pharmacological intervention. Currents with decay times between 21 ms and 23 ms ($21 < \tau < 23$) were not included in further analysis. These decay kinetics are similar to the average kinetics used to isolate AMPA- ($\tau \leq 25$ ms) and GABA-mPSCs ($\tau \geq 30$ ms) in our previous study (Gonzalez-Islas and Wenner, 2006). Events reported in this study have slightly shorter decay times and larger amplitudes for control embryos suggesting differences in space clamp (Rall, 1969).

Statistics.

Data from averages are expressed as mean $\pm$ SEM. Statistical analyses of multiple mPSC amplitudes were performed using ANOVA with post-hoc Tukey's test ($\alpha = 0.05$). Single comparisons were made using Student's t-test ($\alpha = 0.05$). For cumulative distributions, best-fit analysis was performed using a Kolmogorov-Smirnov test ($\alpha = 0.05$).

Remaining Questions and Future Directions

The results from these experiments demonstrate that the $GABA_A$ receptor is a critical step in the sensing machinery that triggers changes in excitatory quantal amplitude after activity perturbations. These are exciting finding because they suggest that $GABA_A$ mediated neurotransmission can regulate the strength of both GABAergic and AMPAergic synapses in a homeostatic manner. Many new questions are raised by these findings. In this section I will outline a few of the most pertinent questions remaining and briefly describe experiments that could be used to evaluate these issues.

Possible mechanisms for the homeostatic scaling of quantal amplitude

Several different mechanisms could be involved in the increase in excitatory quantal amplitude. Increased quantal amplitude could be due to an increase in postsynaptic receptor density or an increase in the amount of neurotransmitter packaged in individual synaptic vesicles. Activity block of cultured central synapses leads to the increased accumulation of postsynaptic glutamatergic receptors. It will be important to test these two possibilities after $GABA_A$ receptor block to determine if similar mechanisms are employed after activity block and after $GABA_A$ receptor block.

Possible mechanisms that could be activated by $GABA_A$ receptor signaling

Interestingly, $GABA_A$ receptor block increased the strength of both GABAergic and AMPAergic synapses. Having one signal that can trigger

homeostatic increases in the strength of all excitatory synapses makes sense. If different signals triggered changes in GABAergic and AMPAergic synapses, then a disconnect could possibly occur which would lead to the increase in one set of excitatory synapses but not the other. This would undermine the goal of coordinately regulating synaptic strength to establish balance. How $GABA_A$ receptor transmission produces the coordinated changes in both GABAergic and AMPAergic synapses is unclear.

In a series of elegant studies, Malenka and colleagues (Beattie et al., 2002; Stellwagen and Malenka, 2006; Kaneko et al., 2008) suggest that glutamate released by synaptic activity is monitored by nearby glial cells. When activity decreases, so does the amount of extracellular glutamate. Glia sense the decrease in glutamate and release TNF-alpha in response. TNF-alpha causes an increase in the accumulation of postsynaptic AMPA receptors, thereby increasing the strength of AMPAergic synapses. In this hippocampal culture system glutamate is the neurotransmitter that is being sensed by glia; however, this may be because GABA is inhibitory in this culture system. It is possible that in developing systems such as the developing chick embryo spinal cord, extracellular GABA could be sensed by glia, thus triggering a similar TNF-alpha induced increase in both AMPA and $GABA_A$ receptors.

It will be important to determine if TNF-alpha plays a role in the regulation of synaptic strength in the *in vivo* developing spinal cord. Based on findings in culture (Stellwagen et al., 2005; Stellwagen and Malenka, 2006), I predict that TNF-alpha will be important in the homeostatic scaling of quantal amplitudes in

the developing spinal cord. A finding that TNF-alpha does not trigger changes in quantal amplitude *in vivo* would be important as this would highlight a critical difference in the signaling machinery identified in producing homeostatic changes in synaptic strength in culture and *in vivo*.

A second possibility is that $GABA_A$ receptor signaling regulates the release of BDNF. During episodes of spontaneous activity, BDNF is released into the extracellular space, and then binds to the TrkB receptor (Berninger et al., 1995; Ba et al., 2005). Blocking the TrkB receptor triggers increases in excitatory synaptic strength and decreases in inhibitory synaptic strength; whereas the exogenous application of BDNF blocks these changes in synaptic strength (Rutherford et al., 1998; Swanwick et al., 2006). These data suggest that BDNF signaling through TrkB receptors could be part of the machinery that triggers homeostatic increases in synaptic strength in response to activity blockade. Although BDNF signaling has been shown to be important in homeostatic synaptic scaling in culture, it is unknown whether BDNF can regulate excitatory synaptic strength *in vivo*.

BDNF signaling may be regulated by $GABA_A$ receptor signaling during early development. Interestingly, Obrietan et al. (Obrietan et al., 2002) have shown that BDNF is involved in a positive feedback loop in which GABA induced release of BDNF causes an increase in presynaptic release probability. A separate study has shown that exogenous BDNF application can increase the GABA producing enzyme glutamic acid decarboxylase (GAD) in the presynaptic cell (Henneberger et al., 2005). These studies suggest that $GABA_A$ receptor

signaling could alter BDNF signaling. We would predict that, similar to the findings in culture, blocking BDNF signaling would mimic the effects of $GABA_A$ receptor block.

Time course of changes in quantal amplitude

In this study, we have shown that increases in quantal amplitude occur between 12 and 48 hours of $GABA_A$ receptor block. The majority of previous studies of cultured central synapses have shown that at least 24 to 48 hours of activity block are necessary to trigger changes in excitatory synaptic strength. However, it is important to examine the trajectory of the increase in synaptic strength to better understand the role that these changes play in the maintenance of network activity. Future studies should measure the amplitudes of AMPAergic and GABAergic quantal currents after 24 and 36 hours of $GABA_A$ receptor block. Determining whether quantal amplitude increases gradually over the period of $GABA_A$ receptor block or is triggered suddenly after a specific duration of block is important to understanding the function of the compensatory changes.

Possible mechanisms that underlie the recovery of SNA

One of the pivotal findings of this study is that *in ovo* activity can occur prior to changes in quantal amplitude. Few studies of homeostatic synaptic strength have been able to examine the recovery of SNA because activity cannot recover due to the chronic administration of the action potential inhibitor TTX.

Although few studies have examined activity recovery, the homeostatic increase in quantal amplitude has been thought to underlie recovery of activity. This is one of the first studies to show that mechanisms other than increases in quantal amplitude are likely to underlie the recovery of activity. Several studies have shown a homeostatic increase in cellular excitability that occurs in response to activity block. $GABA_A$ receptor block could trigger fast increases in cellular excitability that act to recover SNA *in ovo*. This possibility will be examined in detail in chapter 3 of this dissertation.

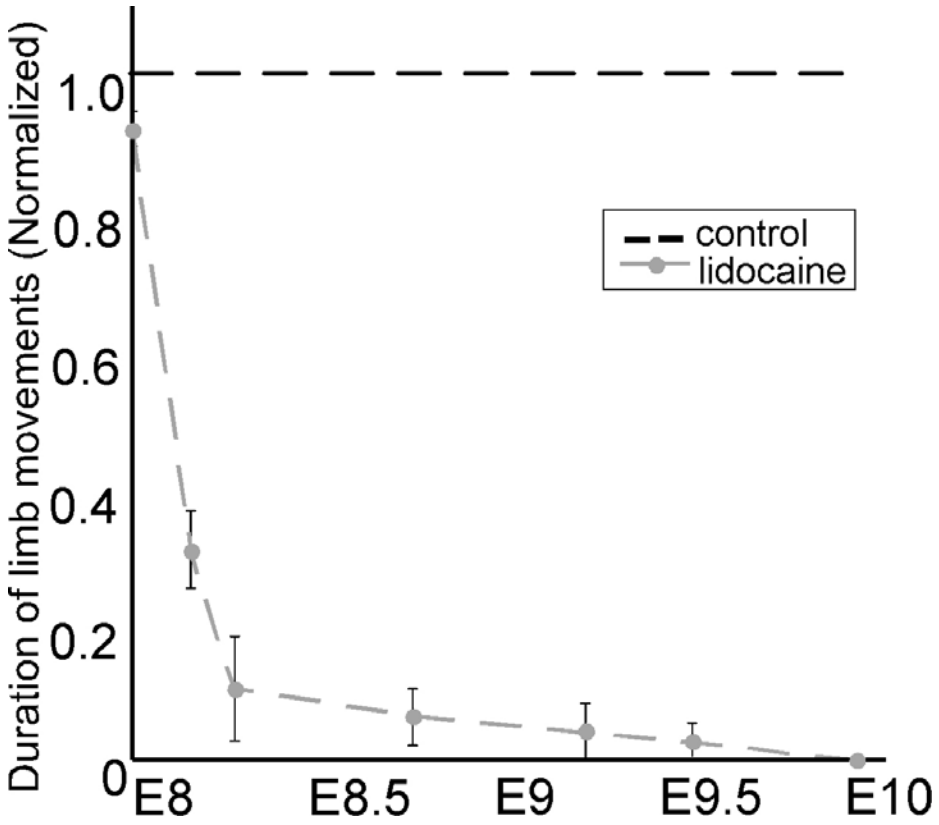

Figure 2.1. Spontaneous network activity is chronically reduced by lidocaine treatment. A graph of the average amount of time chick embryos moved during 5-minute period of observation. Measurements were obtained once every 1-12 hours from E8-E10. Data are normalized to control values (n = 14) at each observation (dotted line). Lidocaine treatment (n = 6) reduced limb movements for approximately 48 hours. As the concentration of lidocaine builds in the egg, the duration of limb movements is decreased. Error bars, SE.

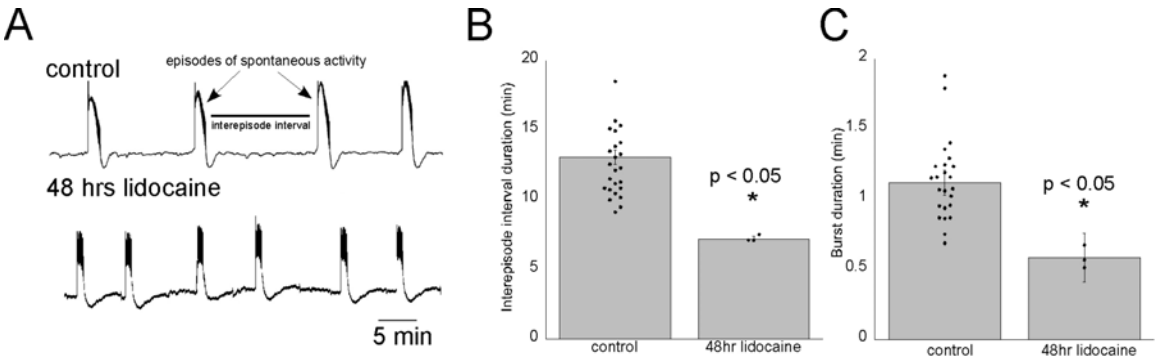

Figure 2.2 Activity block increases SNA frequency *in vitro*.

(A) Representative extracellular recordings from muscle nerves show that lidocaine treatment increases the frequency of episodes of SNA. (B) The duration of the interval between episodes (inter-episode interval) is significantly decreased after lidocaine treatment (n = 3) compared to control (n = 24). (C) The duration of the episodes of SNA is significantly decreased after lidocaine treatment compared to control. Closed circles indicate individual data points. Error bars, SE.

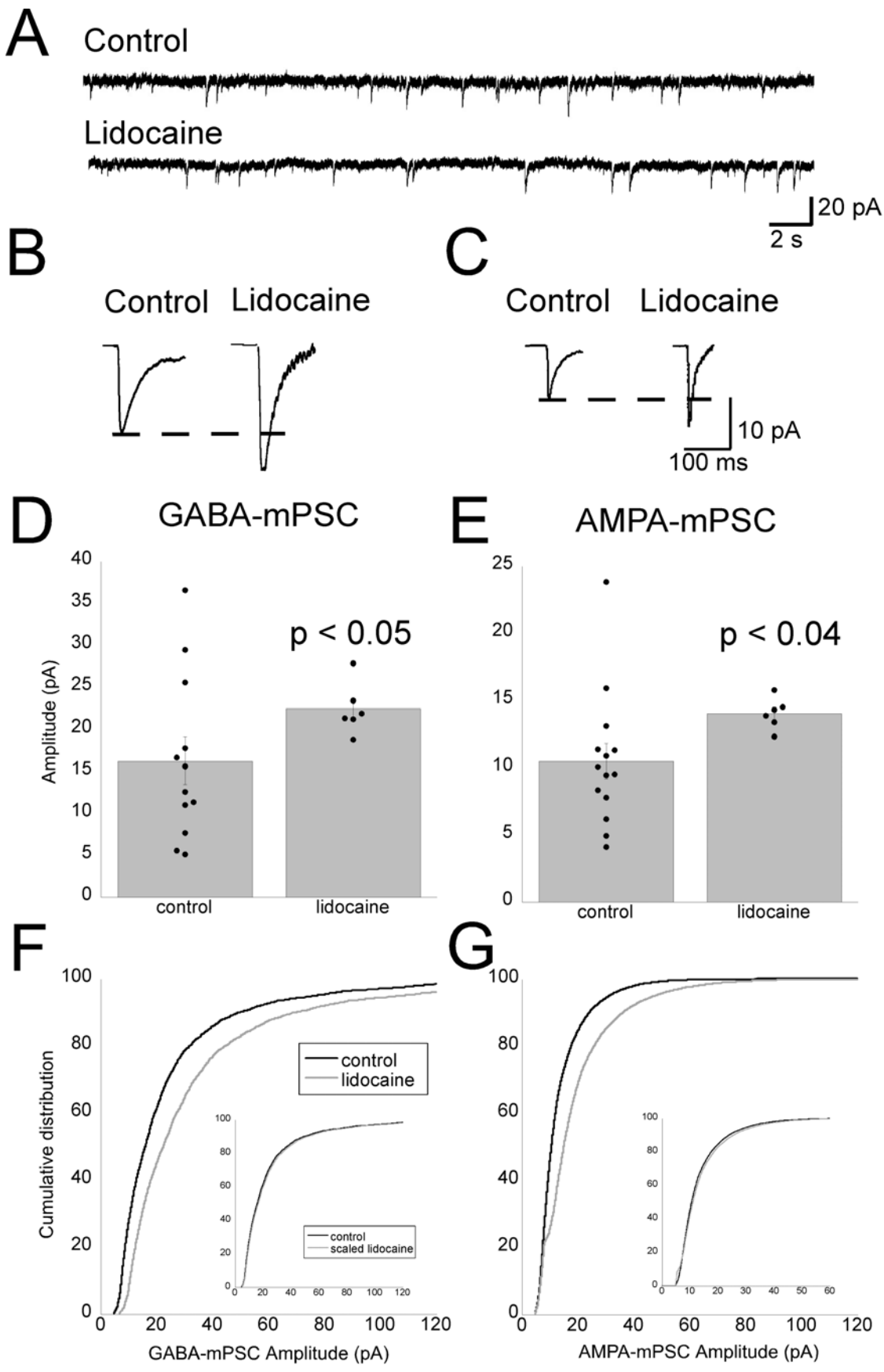
A
Control
Lidocaine
20 pA
2 s
B
Control
Lidocaine
C
Control
Lidocaine
10 pA
100 ms
D
GABA-mPSC
Amplitude (pA)
p < 0.05
control
lidocaine
E
AMPA-mPSC
p < 0.04
control
lidocaine
F
Cumulative distribution
control
lidocaine
control
scaled lidocaine
GABA-mPSC Amplitude (pA)
G
AMPA-mPSC Amplitude (pA)

Figure 2.3. Forty-eight hours of activity block increased the amplitude of GABA- and AMPA-mPSCs. (A) Representative voltage clamp recordings from motoneurons of saline- and lidocaine-treated embryos. (B and C) Average GABA-mPSC (B) and AMPA-mPSC (C) from control and treated embryos. (D and E) The average amplitude of GABA-mPSCs (D) or AMPA-mPSCs (E) was significantly increased in lidocaine-treated embryos ($p < 0.05$, GABA-mPSCs; $p < 0.04$, AMPA-mPSCs). Closed circles indicate individual data points. Error bars, SE. (F and G) Cumulative histogram shows that the entire distribution of GABA-mPSC amplitudes (F) or AMPA-mPSC amplitudes (G) for lidocaine-treated embryos (gray) shifted to the right compared to controls (black). (F inset) The lidocaine distribution could be collapsed onto the control distribution by dividing every point in the distribution by a factor of 1.34 ($p < 0.05$). Amplitudes < 5 pA were excluded from the distributions both before and after scaling the distributions. (G inset) The lidocaine distribution of AMPA-mPSC amplitudes nearly collapsed onto the control distribution after dividing each point by a factor of 1.5 ($p < 0.06$).

Table 2.1. mPSC Amplitudes and Frequencies

GABA - mPSC					
Treatment Condition	n	amplitude (pA)	frequency (Hz)	rise time (ms)	decay time (ms)
Control	14	16.3 ± 2.8	0.3 ± 0.1	12.4 ± 5.3	50.1 ± 6.4
48 hrs lidocaine	6	22.7 ± 1.3 [c]	0.4 ± 0.1	12.8 ± 1.6	52.1 ± 2.3
48 hrs bicuculline	5	33.9 ± 5.2 [a]	0.6 ± 0.2	11.6 ± 1.9	50.2 ± 5.7
48 hrs gabazine	9	29.7 ± 2.6 [a]	0.3 ± 0.1	13.8 ± 5.8	53.6 ± 10.7
12 hrs gabazine	7	17.5 ± 1.9	0.4 ± 0.7	12.7 ± 0.8	51.2 ± 5.5
48 hrs CNQX	6	14.9 ± 1.1	0.4 ± 0.9	14.3 ± 2.1	47.6 ± 3.2
12 hrs CNQX	8	16.5 ± 2.0	0.4 ± 0.7	12.9 ± 4.3	49.5 ± 4.2
48 hrs CNQX + APV	6	15.3 ± 2.3	0.3 ± 0.3	12.2 ± 1.4	41.6 ± 9.5
12 hrs CNQX + APV	7	16.8 ± 2.9	0.4 ± 0.4	12.8 ± 2.5	48.9 ± 8.7

AMPA – mPSC					
Treatment Condition	n	amplitude (pA)	frequency (Hz)	rise time (ms)	decay time (ms)
Control	14	10.5 ± 1.3	0.8 ± 0.2	8.7 ± 5.9	9.3 ± 5.3
48 hrs lidocaine	6	14.1 ± 0.5 [d]	0.8 ± 0.1	9.3 ± 1.2	9.4 ± 1.0
48 hrs bicuculline	5	17.6 ± 2.6 [b]	1.0 ± 0.4	7.5 ± 0.9	8.8 ± 1.2
48 hrs gabazine	9	17.1 ± 1.3 [a]	0.6 ± 0.1	10.5 ± 6.5	7.8 ± 4.6
12 hrs gabazine	7	10.4 ± 0.9	0.8 ± 0.4	11.4 ± 1.7	8.5 ± 0.7
48 hrs CNQX	6	9.3 ± 0.7	0.4 ± 0.1	10.7 ± 1.7	8.1 ± 1.0
12 hrs CNQX	8	9.8 ± 0.5	0.6 ± 0.2	10.1 ± 1.1	9.2 ± 1.0
48 hrs CNQX + APV	6	10.4 ± 1.2	0.4 ± 0.1	10.3 ± 1.6	8.3 ± 0.9
12 hrs CNQX + APV	7	10.3 ± 0.9	0.7 ± 0.3	10.0 ± 1.5	8.9 ± 1.2

n = number of cells recorded; values are mean ± S.E.; One way ANOVA
[a] Statistically significant difference compared to control ($p \leq 0.01$)
[b] Statistically significant difference compared to control ($p \leq 0.03$)
[c] Statistically significant difference compared to control ($p \leq 0.05$)
[d] Statistically significant difference compared to control ($p \leq 0.04$)

Table 2.2. Effect of drug treatment on passive membrane properties

Treatment Condition	n	Membrane Potential (mV)	Input Resistance (MΩ)	Membrane Capacitance (pF)
Control	14	-50.6 ± 0.6	364.1 ± 26.8	21.1 ± 6.8
48hrs lidocaine	6	-51.5 ± 0.4	327.4 ± 21.1	18.7 ± 1.7
48hrs bicuculline	5	-52.5 ± 1.8	368.0 ± 20.4	19.8 ± 3.8
48hrs gabazine	9	-53.6 ± 2.6	366.2 ± 14.1	23.6 ± 7.3
12hrs gabazine	7	-52.3 ± 1.2	337.3 ± 27.2	16.4 ± 2.6
48hrs CNQX	6	-51.7 ± 0.7	329.4 ± 20.9	17.8 ± 0.6
12hrs CNQX	8	-50.9 ± 0.6	388.1 ± 15.0	20.3 ± 1.1
48hrs CNQX + APV	6	-51.3 ± 0.4	377.8 ± 18.4	15.7 ± 1.2
12hrs CNQX + APV	7	-51.9 ± 0.6	383.6 ± 23.7	18.9 ± 1.4

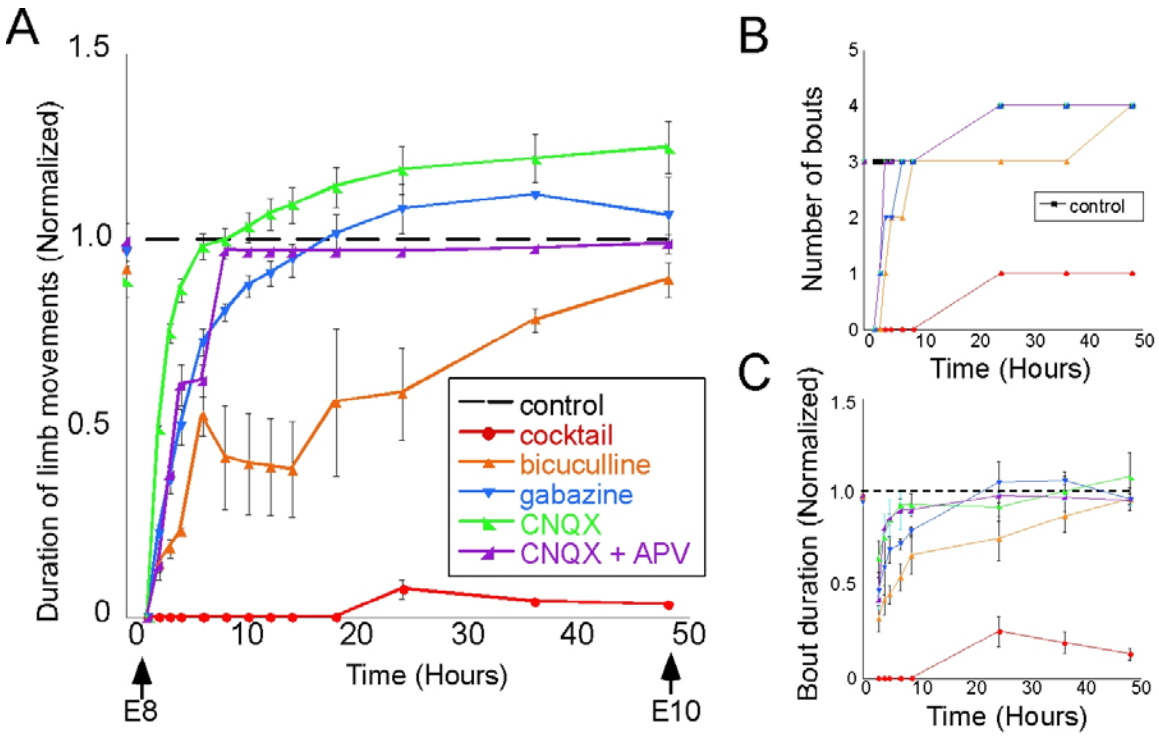

Figure 2.4. GABAergic or glutamatergic receptors can be chronically blocked without a prolonged reduction of spontaneous network activity.

(A) Graph of the average amount of time chick embryos moved during a 5-minute period of observation, obtained once every 0.5-12 hours. Data are normalized to control values at each observation time-point/stage, thus producing dotted line for comparison. Treatment with a single receptor antagonist (bicuculline, n = 4 embryos; gabazine, n = 5; CNQX, n = 3; CNQX + APV, n = 6) transiently reduces embryonic movements; however, movements return to control (n = 13) levels within 12 hours. A cocktail of gabazine, CNQX, strychnine, and APV (n = 9) nearly abolishes limb movements for entire 48 hours. (B) Graph of the median number of bouts of limb movements during a 5-minute period of observation. (C) Graph of the average amount of time chick embryos moved during each movement bout over a 5-minute period of observation. Error bars, SE.

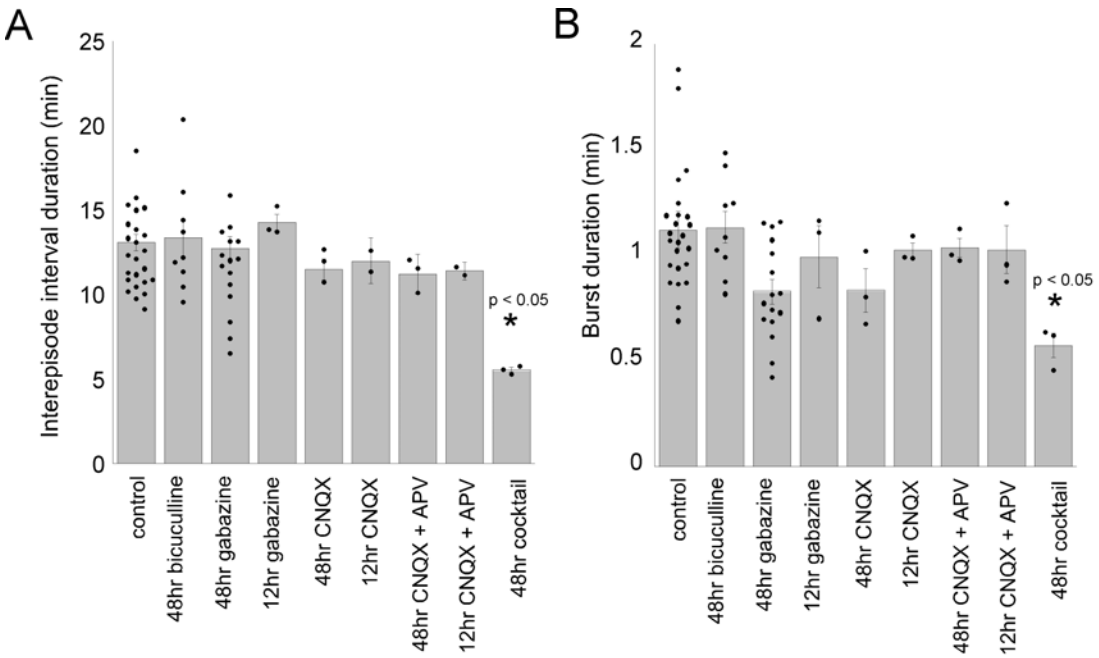

Figure 2.5. Neurotransmitter block does not alter SNA frequency *in vitro*. (A) Bar chart shows that the average interval between episodes (inter-episode interval) is not altered by block of GABAergic (bicuculline and gabazine) or glutamatergic (CNQX and CNQX + APV) transmission for 12 or 48 hours. The block of all neurotransmission (which also blocks SNA *in ovo*) using a cocktail of gabazine, CNQX, APV, and strychnine reduced the inter-episode interval to approximately half of control ($p < 0.05$). This reduction in the time between episodes is similar to the reduction seen after activity block using lidocaine. (B) The duration of the episodes of SNA are not altered by GABAergic or glutamatergic receptor blockade. Similar to activity block using lidocaine, blocking all neurotransmission using a cocktail of antagonists significantly reduces episode duration ($p < 0.05$). Closed circles represent individual data points. Error bars, SE.

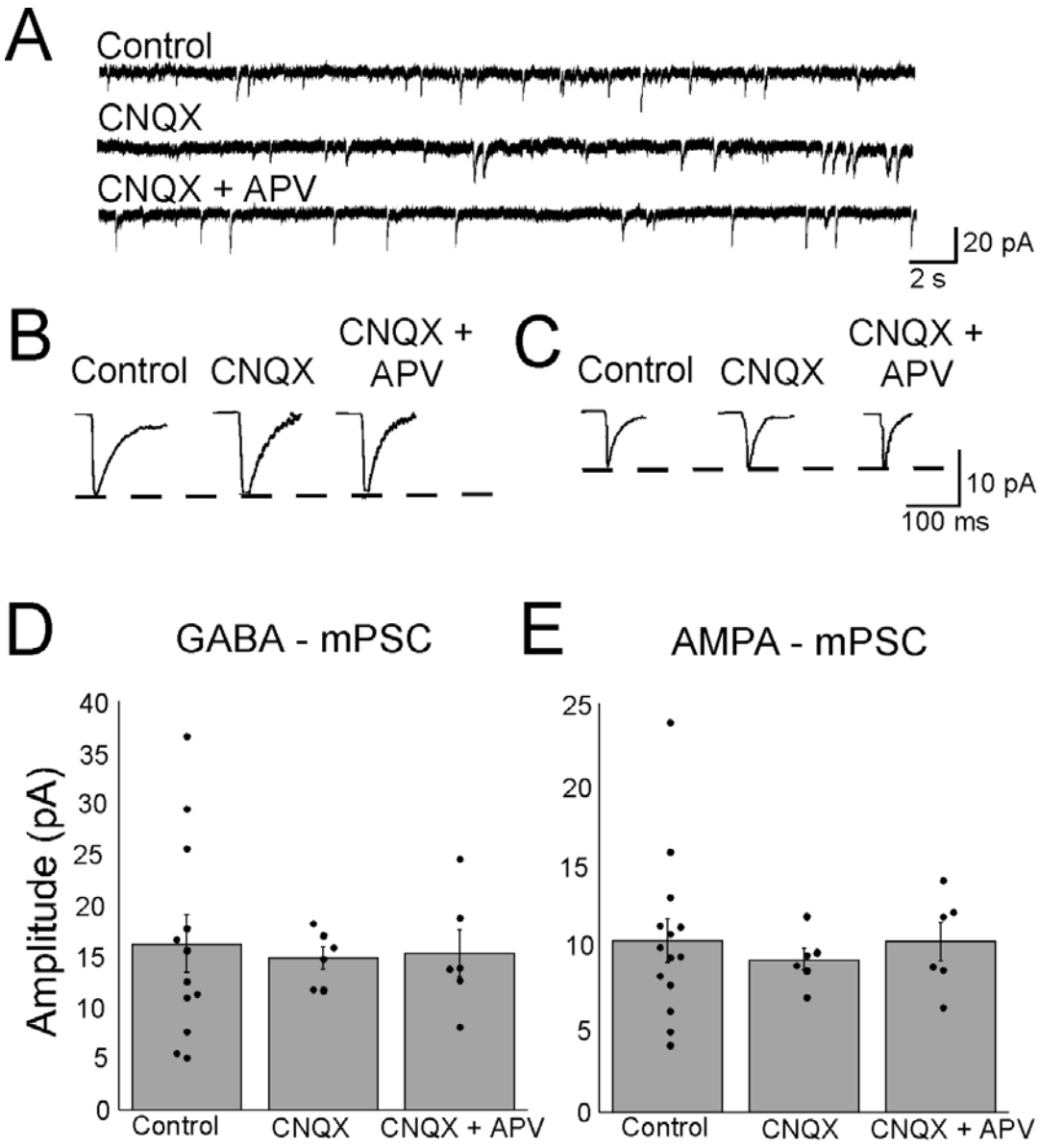

Figure 2.6. 48 hours of ionotropic glutamate receptor blockade did not alter the amplitude of GABA- or AMPA-mPSCs. (A) Representative voltage clamp recordings from motoneurons of saline-, CNQX-, or CNQX + APV-treated embryos. Average GABA-mPSC (B) or AMPA-mPSC (C) from saline-, CNQX-, and CNQX + APV-treated embryos. Treatment with CNQX or CNQX + APV from E8-10 did not significantly alter the average amplitude of GABA-mPSCs (D) or AMPA-mPSCs (E) compared to control. Closed circles represent individual data points. Error bars, SE.

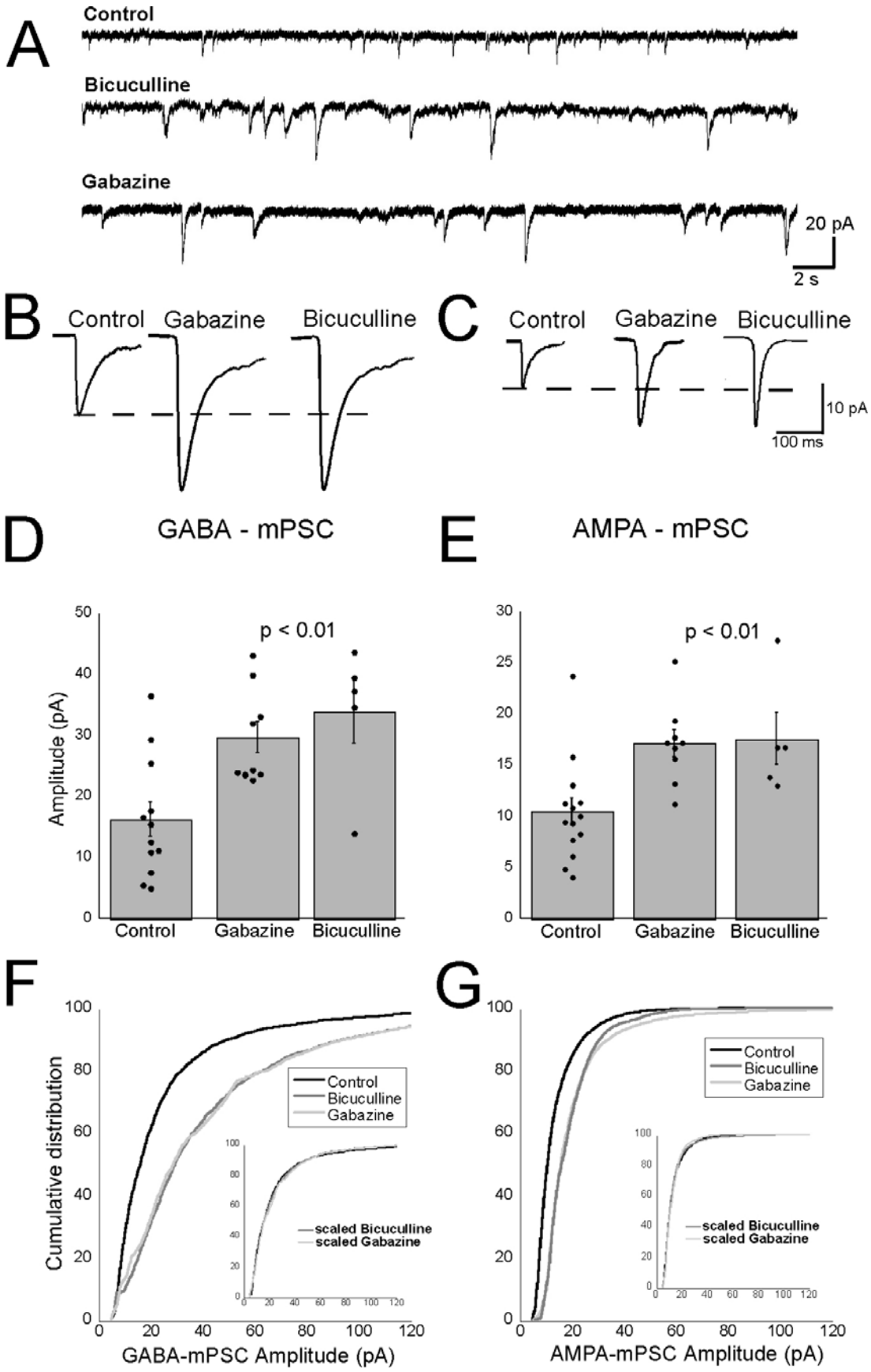
A
Control
Bicuculline
Gabazine
20 pA
2 s
B
Control
Gabazine
Bicuculline
C
Control
Gabazine
Bicuculline
10 pA
100 ms
D
GABA - mPSC
p < 0.01
Amplitude (pA)
Control
Gabazine
Bicuculline
E
AMPA - mPSC
p < 0.01
Control
Gabazine
Bicuculline
F
Cumulative distribution
Control
Bicuculline
Gabazine
scaled Bicuculline
scaled Gabazine
GABA-mPSC Amplitude (pA)
G
Control
Bicuculline
Gabazine
scaled Bicuculline
scaled Gabazine
AMPA-mPSC Amplitude (pA)

Figure 2.7. 48 hours of $GABA_A$ receptor blockade increased the amplitude of GABA- and AMPA-mPSCs. (A) Representative voltage clamp recording from motoneurons of saline-, bicuculline-, and gabazine-treated embryos. Average GABA-mPSC (B) or AMPA-mPSC (C) from saline-, gabazine-, and bicuculline-treated embryos. The average amplitude of GABA-mPSCs (D) or AMPA-mPSCs (E) was significantly increased in bicuculline- and gabazine-treated motoneurons compared to control. Closed circles indicate individual data points. Error bars, SE. (F) Cumulative histogram shows that the entire distribution of GABA-mPSC amplitudes for motoneurons from bicuculline- (dark gray) and gabazine-treated (light gray) embryos shifted to the right. The bicuculline and gabazine distributions are similar ($p = 0.01$). (F, inset) The bicuculline and gabazine distributions could be collapsed onto the control distribution by dividing every point in the distribution by a factor of 2.0 ($p = 0.03$), suggesting they scale. Amplitudes less than 5 pA were excluded from the distributions both before and after scaling the distributions. (G) Cumulative histogram shows that the entire distribution of AMPA-mPSC amplitudes for motoneurons from bicuculline- (dark gray) and gabazine-treated (light gray) embryos shift to the right compared to control (black), again suggesting the entire distribution is scaled. The bicuculline and gabazine distributions are similar ($p = 0.03$). (G, inset) The bicuculline distributions could be collapsed onto the control distribution by dividing every point in the distribution by a factor of 1.5 ($p = 0.03$). The gabazine distribution nearly collapsed onto the control distribution after dividing each point by a factor of 1.25 ($p = 0.07$).

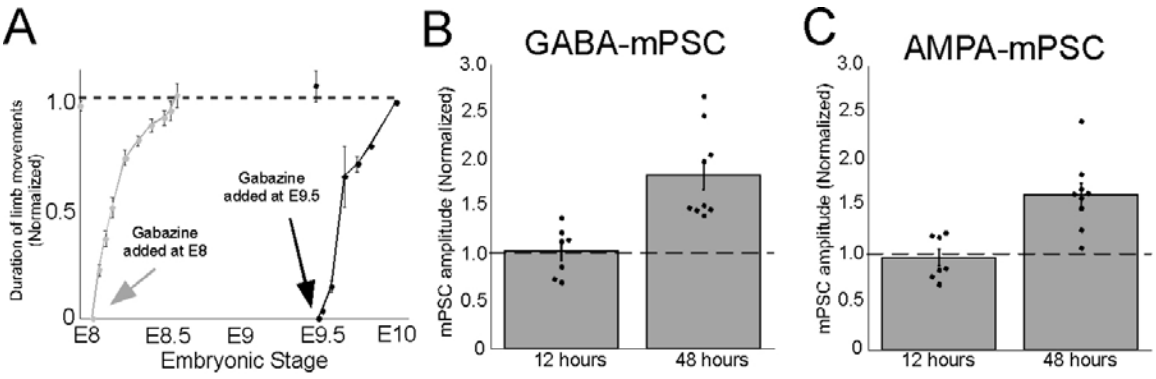

Figure 2.8. 12 hours of $GABA_A$ receptor blockade did not alter the amplitude of GABA- or AMPA-mPSCs. (A) Graph of the average amount of time chick embryos moved limbs during a 5- minute period of observation. Data is normalized to control values at each time-point in which observations were collected. Blocking $GABA_A$ receptors *in ovo* (gabazine) for 12 hours, beginning at E8 or at E9.5, transiently reduces the embryonic limb movements, which recover within 12 hours. (B) The average amplitude of GABA-mPSCs is not significantly different among motoneurons from embryos treated with saline (control) or gabazine for 12 hours. Gabazine treatment for 48 hours significantly increased the amplitude of GABA-mPSCs compared to control (48 hour data same as that in Figure 3D). (C) The average amplitude of AMPA-mPSCs is not significantly different among motoneurons from embryos treated with saline (control) or gabazine for 12 hours. Gabazine treatment for 48 hours significantly increased the amplitude of AMPA-mPSCs compared to control (48 hour data same as that in Figure 3E). Dashed lines represent control values. Error bars, SE.

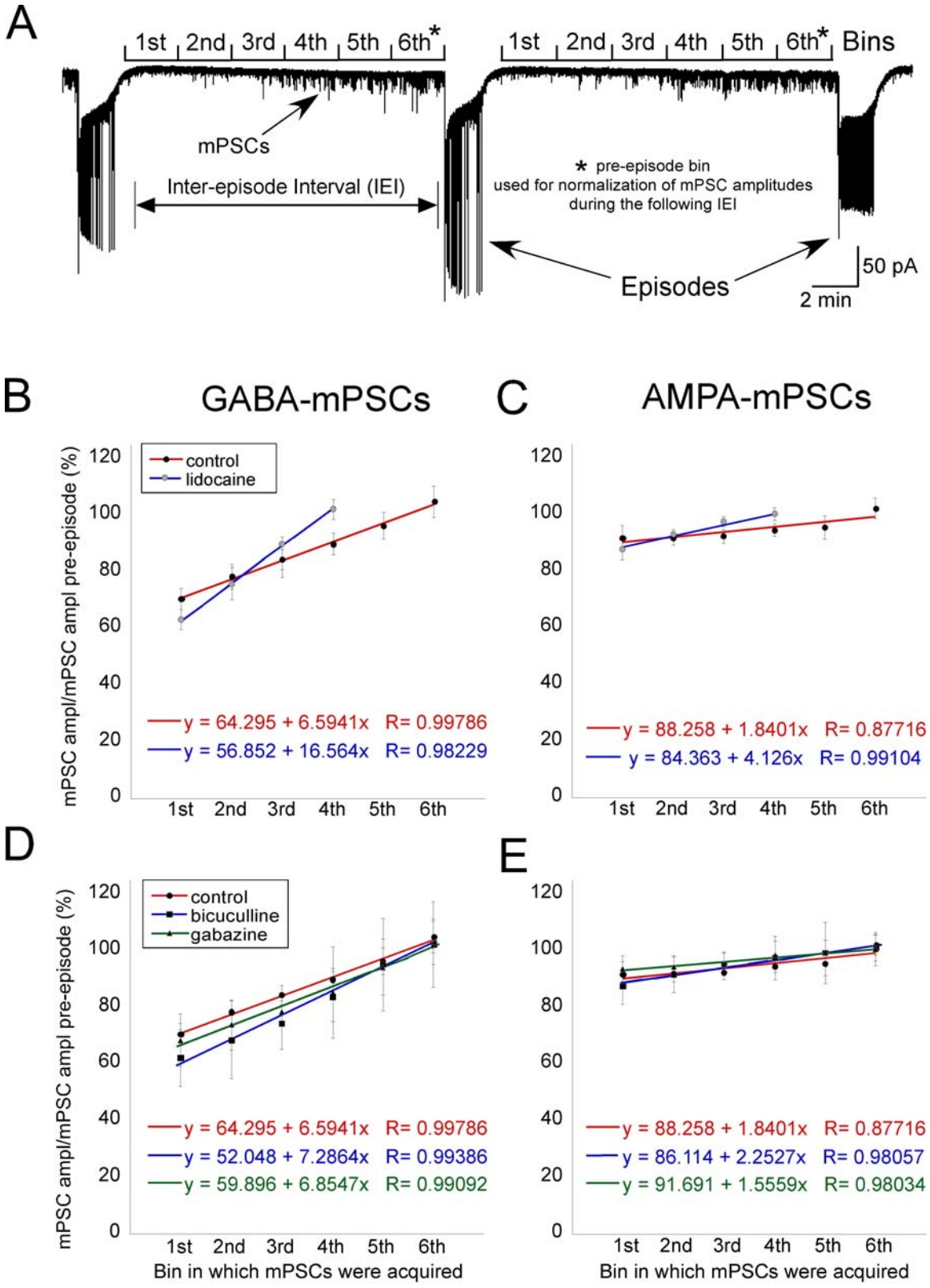
A
1st 2nd 3rd 4th 5th 6th* 1st 2nd 3rd 4th 5th 6th* Bins
mPSCs
Inter-episode Interval (IEI)
* pre-episode bin
used for normalization of mPSC amplitudes
during the following IEI
Episodes
50 pA
2 min
B
GABA-mPSCs
C
AMPA-mPSCs
mPSC ampl/mPSC ampl pre-episode (%)
control
lidocaine
y = 64.295 + 6.5941x R= 0.99786
y = 56.852 + 16.564x R= 0.98229
y = 88.258 + 1.8401x R= 0.87716
y = 84.363 + 4.126x R= 0.99104
1st 2nd 3rd 4th 5th 6th
D
E
control
bicuculline
gabazine
y = 64.295 + 6.5941x R= 0.99786
y = 52.048 + 7.2864x R= 0.99386
y = 59.896 + 6.8547x R= 0.99092
y = 88.258 + 1.8401x R= 0.87716
y = 86.114 + 2.2527x R= 0.98057
y = 91.691 + 1.5559x R= 0.98034
Bin in which mPSCs were acquired

Figure 2.9. Activity block, but not $GABA_A$ receptor block, accelerates recovery from post-episode depression. (A) Schematic indicating the process of separating mPSCs into 2-minute bins to assess the modulation of mPSC amplitudes after episodes of SNA. (B-E) The average amplitude of mPSCs in consecutive 2-minute bins (starting 30 s after the episode) as a percentage of the value in the last 2-minute bin just before the episode. (B) The average GABA-mPSC amplitude is depressed to approximately the same percentage after the episode in both control and lidocaine-treated embryos. However, the line fit (equation shown) indicates that the amplitudes of GABA-mPSCs from lidocaine-treated embryos (blue line; n = 6) recover faster from depression than amplitudes of GABA-mPSCs from control embryos (red line; n = 14). (C) AMPA-mPSC amplitudes do not exhibit significant post-episode depression or modulation of amplitude during the inter-episode interval. (D) The average amplitude of GABA-mPSCs from saline-, bicuculline-, and gabazine-treated embryos is reduced to approximately 60% of pre-episode values in the first 2-minute bin after the episode. There is no significant difference in post-episode depression of GABA-mPSC amplitudes among saline-, gabazine-, or bicuculline-treated embryos. The best-fit line equations show no significant difference in slope among treatment groups suggesting that the recovery of GABA-mPSC amplitudes is not altered by $GABA_A$ receptor block (bicuculline, blue, n = 5; gabazine, green, n = 9; control, red, n = 14). (E) AMPA-mPSC amplitudes do not undergo modulation during the inter-episode interval.

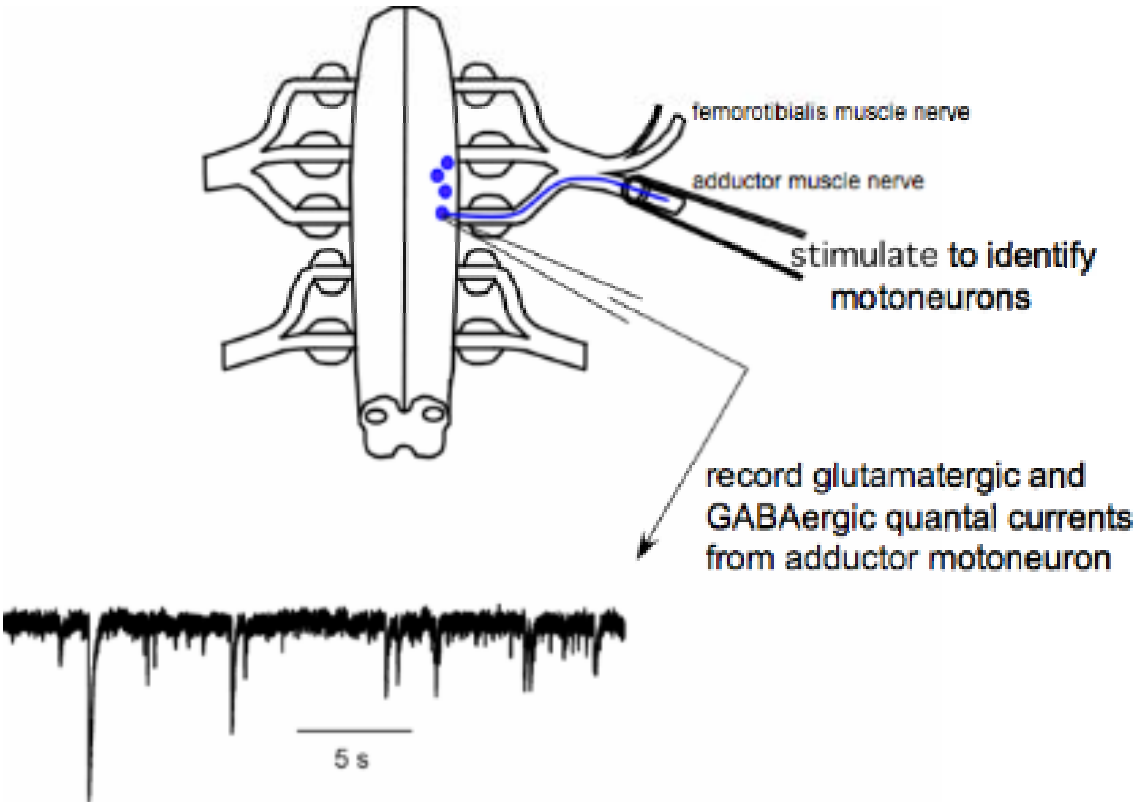

Figure 2.10. The isolated spinal cord is maintained *in vitro* for all whole cell recordings. Suction electrodes attached to the intact adductor or femorotibialis muscle nerves are used to antidromically identify motoneurons. GABAergic and glutamatergic quantal currents can be monitored using whole cell voltage clamp.

Table 2.3. Spontaneous postsynaptic current (sPSC) amplitudes and frequencies are not affected by acute TTX treatment. Thus, we define recorded sPSCs as miniature postsynaptic currents (mPSCs).

Treatment Condition	n	amplitude (pA)	frequency (Hz)	rise time (ms)	decay time (ms)
GABA mPSC-Control	14	16.3 ± 2.8	0.3 ± 0.1	12.4 ± 5.3	50.1 ± 6.4
GABA mPSC-Acute TTX	3	19.2 ± 3.6	0.3 ± 0.1	13.7 ± 5.8	50.6 ± 4.7
AMPA mPSC-Control	14	10.5 ± 1.3	0.8 ± 0.2	8.7 ± 5.9	9.30 ± 5.3
AMPA mPSC-Acute TTX	3	10.6 ± 1.1	0.8 ± 0.4	9.2 ± 4.7	9.15 ± 4.1

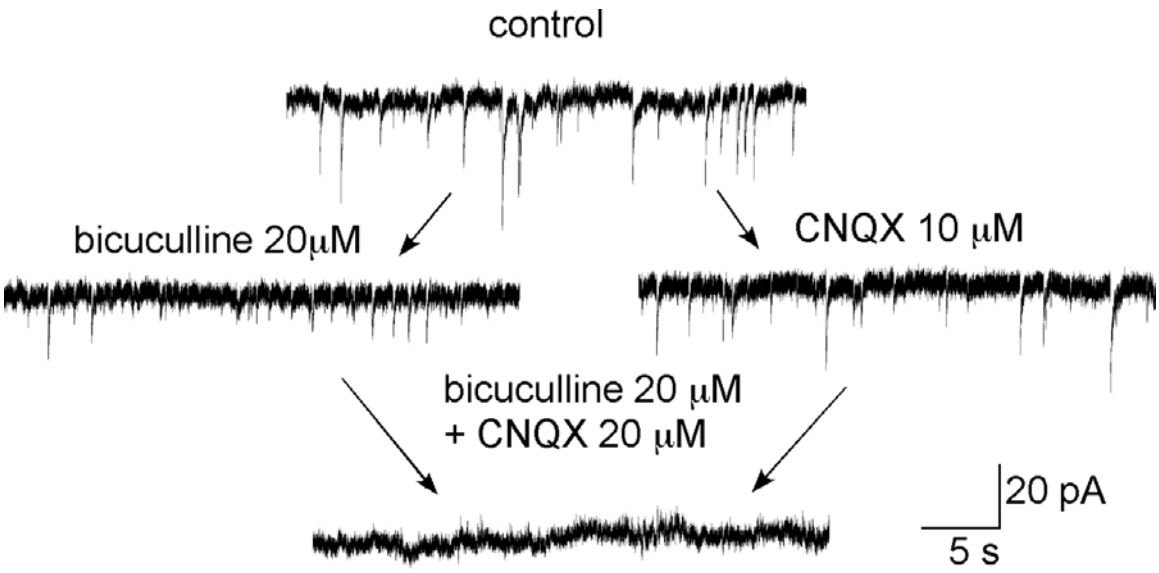

Figure 2.11. Identification of populations of mPSCs in motoneurons. AMPAergic mPSCs are isolated by bicuculline; GABAergic mPSCs are isolated by CNQX. The addition of bicuculline and CNQX blocks virtually all mPSCs. No NMDA currents are recorded because all recordings are performed at a holding potential of -70 mV.

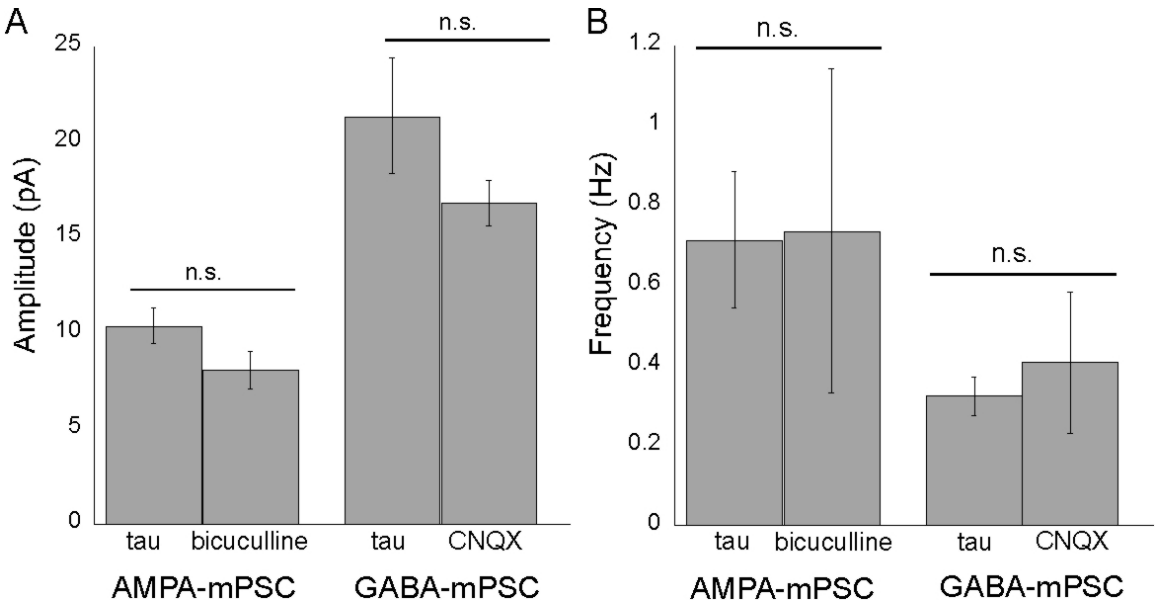

Figure 2.12. Kinetically isolated mPSCs are similar to those isolated pharmacologically. Average mPSC amplitude (A) and frequency (B) are similar for mPSCs isolated by decay kinetics (tau) or isolated by treatment with receptor antagonists. AMPA-mPSCs are isolated using bicuculline (n=3); GABA-mPSCs are isolated using CNQX (n=3). Error bars, SE.

CHAPTER 3:

HOMEOSTATIC RECOVERY OF SPONTANEOUS NETWORK ACTIVITY THROUGH COMPENSATORY CHANGES IN CELLULAR EXCITABILITY

Modified from the following reference:

Wilhelm, Jennifer C., Rich, Mark M., and Wenner, Peter A. (2008). Homeostatic recovery of network activity through compensatory changes in cellular excitability. (submitted).

Abstract

When neural activity is reduced over a period of days homeostatic changes in synaptic strength and/or cellular excitability are triggered, which are thought to act in a manner to recover normal activity levels. The time course over which changes in homeostatic synaptic strength and cellular excitability occur are not clear. Although many studies show that 1-2 days of activity block are necessary to trigger increases in excitatory quantal strength, few studies have been able to examine whether these mechanisms actually underlie recovery of network activity. Here we examine the mechanisms underlying recovery of activity following block of either excitatory GABAergic or glutamatergic inputs *in ovo*. We find that $GABA_A$ receptor blockade triggers fast changes in cellular excitability that occur prior to changes in synaptic strength. This increase in cellular excitability, mediated in part by increase in sodium currents and the reduction in the fast-inactivating and calcium-activated potassium currents, could underlie the recovery of normal activity levels *in ovo*. These findings suggest an important role for cellular excitability in recovering activity as well as a special role for the $GABA_A$ receptor in triggering changes in both synaptic strength and cellular excitability after activity perturbations. The temporal difference in expression of homeostatic changes in cellular excitability and synaptic strength suggest that there are multiple mechanisms and pathways engaged to regulate network activity and they may have temporally distinct functions.

Introduction

During development many synaptic networks exhibit spontaneous network activity (SNA), which is thought to be important in the maturation of these synaptic networks (Feller, 1999; O'Donovan, 1999; Ben-Ari, 2001). In the spinal cord, the vast majority of neurons are synchronously activated during these episodic bursts of SNA, which are separated by periods of quiescence. SNA is produced by hyperexcitable, recurrently connected circuits in which both GABA and glutamate are excitatory. Spinal SNA has been shown to be important for various aspects of limb (O'Donovan et al., 1998b) and motoneuron development (Casavant et al., 2004; Hanson and Landmesser, 2004). We have recently shown that SNA appears to be important in the development of synaptic strength within the networks that generate it (Gonzalez-Islas and Wenner, 2006). When SNA was reduced for 48 hours *in vivo* compensatory increases in AMPA and $GABA_A$ quantal amplitude were observed. These compensatory changes in synaptic strength have been shown in many different systems and are thought to contribute to the homeostatic recovery and maintenance of network activity levels (Rich and Wenner, 2007; Turrigiano, 2007).

The machinery by which cells or networks sense activity perturbations and then trigger changes in synaptic strength is less clear. In the embryonic spinal cord, GABAergic neurotransmission is an important part of the sensing machinery that triggers changes in synaptic strength (Wilhelm and Wenner, 2008). This was demonstrated by injecting a $GABA_A$ receptor antagonist *in ovo*

to block GABAergic signaling for 48 hours. The addition of the antagonist *in ovo* produced a transient reduction in SNA that recovered to control levels within 12 hours. Interestingly, this activity recovery occurred prior to changes in quantal amplitude, suggesting the recovery was dependent on mechanisms other than changes in quantal strength.

Although it is often assumed that the compensatory changes in quantal amplitude underlie the homeostatic recovery of activity, little is actually known about the time course of the mechanisms that are thought to be responsible for this recovery (Wilhelm and Wenner, 2008). Comparing the time course of the recovery process and that of the compensatory mechanisms triggered by activity perturbations is critical to understanding how activity functionally recovers and is then maintained. Several studies have shown that blocking network activity produces compensatory increases in cellular excitability in addition to changes in synaptic strength (Desai, 2003). Previous work suggests that changes in cellular excitability may occur more quickly than changes in quantal amplitude (Aptowicz et al., 2004; Karmarkar and Buonomano, 2006). These changes in cellular excitability could contribute to the recovery of activity, and may even allow full recovery before compensatory changes in quantal amplitude are engaged.

In this study, we show that blocking $GABA_A$ receptor signaling for 12 hours in the developing spinal cord *in vivo* triggers compensatory changes in cellular excitability. These changes in cellular excitability could account for the recovery of SNA, which also occurs within 12 hours. Quantal amplitude changes only after 12 hours, suggesting this form of synaptic strength did not contribute to the

recovery. Changes in cellular excitability were not mediated by voltage gated calcium channels or delayed rectifier like channels, but rather by reductions in transient and calcium dependent potassium channel currents.

Results:

$GABA_A$ receptor block increases intrinsic excitability

Blocking $GABA_A$ transmission reduced embryonic movements (our measure of SNA), which recovered within 12 hours; before compensatory increases in quantal amplitude were observed [Figure 3.1A; (Wilhelm and Wenner, 2008)]. To determine if cellular excitability contributed to the recovery of activity after $GABA_A$ receptor blockade, we treated embryos *in ovo* for either 12 hours (E9.5 – E10) or 48 hours (E8 - E10) with saline (1 ml), the glutamate receptor antagonists CNQX (10 μM; assume 50 ml egg volume) and APV (100 μM), or the $GABA_A$ receptor antagonist gabazine (20 μM). Gabazine, CNQX+ APV, or saline were applied to the chorioallantoic membrane through a small window in the shell. At E10, spinal cords were isolated from the embryos and whole cell current clamp recordings were obtained from antidromically identified motoneurons. Cellular excitability was examined in two ways: rheobase current and spike threshold. Rheobase current was determined as the least current necessary to produce an action potential (1 pA steps, 500 ms duration). Spike threshold was measured as the difference in voltage between resting membrane voltage (V_m) and the threshold voltage (V_T, voltage evoked by the rheobase current).

$GABA_A$ receptor blockade significantly decreased rheobase current (Figure 3.1B; Table 3.1) and spike threshold (Figure 3.1C, Table 3.1) in the first 12 hours. After 48 hours of $GABA_A$ receptor blockade both rheobase current (Figure 3.1B; Table 3.1) and spike threshold (Figure 3.1C, Table 3.1) began to return towards control levels. These results suggest that $GABA_A$ receptor blockade can trigger changes in cellular excitability that could contribute to the homeostatic recovery of activity. Further, the changes in cellular excitability occur before changes in synaptic strength (Wilhelm and Wenner, 2008).

We previously demonstrated that blocking ionotropic glutamate receptor signaling also produces a transient decrease in SNA *in ovo* that recovers to control levels at the same rate as $GABA_A$ receptor blockade (Figure 3.1A). However, ionotropic glutamatergic receptor blockade did not result in a change in rheobase current (Figure 3.1B, Table 3.1) or spike threshold (Figure 3.1C, Table 3.1) regardless of the duration of blockade. These results suggest that changes in cellular excitability are not triggered by neurotransmission block in general or the transient activity reduction after antagonist injection, because glutamate receptor blockade did not trigger changes in cellular excitability.

As 12 hours of gabazine treatment produced the largest changes in rheobase currents and spike threshold, we examine the firing rates of motoneurons from embryos treated with saline or 12 hours of gabazine. Average frequency versus current (f-I) curves were constructed by measuring the instantaneous firing frequency (the inverse period between first and second spikes) for each current injection. Gabazine-treated motoneurons exhibited a

leftward shift in the f-I curve, suggesting that $GABA_A$ receptor block increases the sensitivity of motoneurons to current input (Figure 3.1D-E). Also, the slope of the initial, linear part of the f-I curve was greater for gabazine-treated motoneurons (0.34 ± 0.03 Hz/pA, n = 8; control 0.26 ± 0.01 Hz/pA, n = 5; $p \leq 0.05$).

In addition, gabazine treatment increased the half-width of the average action potential (control, 3.1 ± 0.1 ms, n = 8; gabazine, 5.7 ± 0.2 ms, n =8; $p \leq 0.001$; Figure 3.1F) as well as decreasing the peak of after-hyperpolarization (control, -6.9 ± 0.4 mV; gabazine -5.5 ± 0.3 mV; $p \leq 0.05$). The peak of the action potential (control, 39.6 ± 0.5 mV; gabazine, 39.3 ± 0.4 mV) and the rise-time (control 1.5 ± 0.3 ms; gabazine 1.3 ± 0.4 ms) were not altered by gabazine treatment. Together, these findings suggest that $GABA_A$ receptor block increases intrinsic excitability in motoneurons.

Changes in intrinsic cellular excitability could be due to changes in passive membrane properties or voltage-gated ion channels (Desai, 2003). We found that $GABA_A$ receptor blockade-induced changes in excitability were not mediated by changes in passive membrane properties, as gabazine treatment did not affect cellular capacitance, membrane resistance, or resting potential of the neurons (Table 3.1).

$GABA_A$ receptor block increases voltage-activated sodium currents.

As passive membrane properties remain unchanged after gabazine treatment, we hypothesized that gabazine-dependent changes in cellular excitability are mediated by changes in voltage-gated channels as described for

other systems (LeMasson et al., 1993; Turrigiano et al., 1994; Turrigiano et al., 1995; Desai et al., 1999a; Golowasch et al., 1999). To test this, we examined several voltage-activated sodium, calcium, and potassium currents using whole cell voltage clamp recordings from motoneurons of embryos treated for 12 hours with saline (1 ml; control) or gabazine (20 μM) as this produced the largest changes in rheobase current. Although $GABA_A$ receptor blockade is performed for 12 hours *in vivo*, all measurements of currents are performed in the isolated *in vitro* spinal cord.

Using whole cell voltage clamp, sodium currents (I_{Na}) were elicited using voltage steps from a holding potential of -60 mV. Currents were isolated using intracellular and extracellular potassium and calcium channel blockers (see Methods). Because I_{Na} is such a large and fast current, we could not generate a reliable activation curve. However, we were able to detect a 92% increase in the peak sodium current after gabazine treatment when stepping from -60mV to -10mV ($p \leq 0.001$; Figure 3.2). These currents were almost completely blocked by the addition of the sodium channel antagonist tetrodotoxin (TTX; 1 μM; data not shown), suggesting that the isolated currents are sodium channel mediated. These results suggest that $GABA_A$ receptor block increases sodium currents. This increase in the sodium current could underlie the significant increase in cellular excitability as well as the increase in the half-width of the action potential found after $GABA_A$ receptor block.

$GABA_A$ receptor block does not alter voltage-activated calcium currents.

Calcium currents were measured by applying depolarizing voltage steps to +50 mV (in 10 mV increments) from a holding potential of -60 mV. Sodium currents were blocked by 1 μM TTX; potassium currents were blocked by a combination of internal cesium chloride and TEA-Cl and external TEA-Cl and 4-AP. Contribution of remaining currents were significantly reduced by incubation in a Ca^{2+}-free solution (0 mM Ca^{2+}). Net calcium currents were determined by subtracting currents elicited in the absence of external calcium from corresponding currents when calcium was present (10 mM Ca^{2+} - 0 mM Ca^{2+}; Figure 3). The depolarizing voltage steps produced inward currents that peaked within a few milliseconds and decayed toward a sustained current (Figure 3.3A-B). Gabazine treatment did not significantly alter the peak calcium currents when measured from baseline to peak (Figure 3.3C) or as the difference between peak and steady state (data not shown). Additionally, sustained calcium currents, measured 700 ms after the start of the voltage step, were not altered by gabazine treatment (Figure 3.3D). This suggested that voltage activated calcium currents did not contribute to the increased cellular excitability following gabazine treatment.

$GABA_A$ receptor block decreased I_A and $I_{K(Ca)}$.

Outward K^+ currents consisted of two distinct components; a fast-inactivating, TEA-insensitive current (I_A) and a sustained current (I_K) likely composed of Ca^{2+}-activated currents (K_{Ca}) and delayed rectifier currents (K_{dr}).

These currents were recorded in the presence of TTX; however, Ca^{2+} currents were not blocked in order to maintain Ca^{2+}-activated K^+ currents.

Applying depolarizing voltage steps from a holding potential of -100 mV to between -30 mV and +50 mV (in 10 mV increments) elicited large outward K^+ currents consisting of I_A and I_K (Figure 3.4A). I_K could be isolated from I_A when the same voltage steps were preceded by a brief (100 ms) pre-pulse to -40 mV (Figure 3.4B), which inactivates I_A in embryonic chick lumbar motoneurons (McCobb et al., 1990; Martin-Caraballo and Dryer, 2002). I_A was measured as the peak current resulting from the subtraction of I_K from the total K^+ current (Figure 3.4C). The addition of external TEA to block I_K did not alter the peak I_A currents measured suggesting that we have isolated the TEA-insensitive I_A (control, 4.00 ± 0.03 nA; control + TEA, 4.03 ± 0.07 nA; for steps to +50 mV). Gabazine treatment significantly reduced peak A-currents (Figure 3.4D-E) in addition to reducing I_A inactivation kinetics (Figure 3.4F; $p \leq 0.04$). These changes in I_A would tend to increase cellular excitability and might explain the widened action potential. No change was found in the voltage dependence of I_A (Figure 3.4G).

Motoneurons treated for 12 hours with gabazine had significantly reduced I_K compared to currents from control motoneurons (Figure 3.5 A-B). Because I_K is composed of $I_{K(Ca)}$ and $I_{K(dr)}$, we assed gabazine treatment alters $I_{K(Ca)}$ or $I_{K(dr)}$, we used whole cell voltage clamp to apply depolarizing steps to +50 mV (in 10 mV increments) from a holding potential of -40 mV (to inactivate I_A currents). Currents were recorded in a saline solution containing external Ca^{2+} and a Ca^{2+}-

free solution (0 mM Ca^{2+}; Figure 3.5B). $I_{K(Ca)}$ was obtained by digital subtraction (Ca^2 – 0 mM Ca^{2+}; Figure 3.5C). Using this procedure allows the measurement of K_{Ca} currents independently of other currents (McCobb et al., 1990; Martin-Caraballo and Dryer, 2002). Gabazine treatment significantly reduced $I_{K(Ca)}$ (Figure 3.5D). $I_{K(Ca)}$ appears to slightly rectify at the most depolarized step potentials; thus, we did not fit a Boltzmann equation to the activation curves. However, a plot of the mean G/G_{max} values shows considerable overlap between control and gabazine-treated curves (Figure 3.5E), suggesting that the activation of $I_{K(Ca)}$ is not altered after gabazine treatment. As I_{Ca} was not altered by gabazine treatment (Figure 3.3), the reduction in $I_{K(Ca)}$ in gabazine-treated motoneurons cannot be attributed to a change in Ca^{2+} currents. The decrease in $I_{K(Ca)}$ could explain the reduction in after-hyperpolarization found in action potentials after gabazine treatment. $I_{K(dr)}$ was measured as the amount of current elicited in the Ca^{2+}-free solution (Figure 3.5B). Removing Ca^{2+} from the external solution allows for measurement of K_{dr} without a contribution from Ca^{2+}-dependent K^+ currents (McCobb et al., 1990; Martin-Caraballo and Dryer, 2002). Gabazine treatment did not significantly alter peak K_{dr} currents (Figure 3.5F).

$GABA_A$ receptor block differentially regulates ionic current densities.

Individual current measurements were normalized by capacitance before averaging to attain the density of each current. Inward and outward currents were differentially regulated by gabazine-treatment such that I_{Na} was increased to 178% ($p \leq 0.001$), I_A was reduced to 74% ($p \leq 0.04$), $I_{K(Ca)}$ was reduced to 51% ($p \leq 0.001$) compared to control, while I_{Ca} and $I_{K(dr)}$ remained unaltered (Figure 3.6).

These changes in current density could be due to a change in the number of channels, or changes due to properties of single channels, however no change was observed in the activation kinetics of I_A or $I_{K(Ca)}$.

Potassium current densities do not appear to co-regulate.

A recent study showed that removal of neuromodulatory input from neurons in the crustacean STG triggers a process that alters that regulation of current densities such that the density of certain voltage-gated currents that normally varied in a coordinated manner no longer co-vary (Khorkova and Golowasch, 2007). To examine whether the block of $GABA_A$ receptor signaling triggered a similar process, we plotted the densities of I_A versus $I_{K(Ca)}$ and $I_{K(dr)}$ (Figure 3.7). Only potassium currents were examined because I_{Na}, $I_{K(Ca)}$, $I_{K(dr)}$, I_A, and I_{Ca} could not be isolated in the same neurons due to differences in current isolation protocols. We found that when we plotted the density of I_A versus the density of $I_{K(Ca)}$ (with density pairs recorded from the same cell), a correlation was found in controls (Figure 3.7A). However this correlation appeared to be due to the presence of a data point several standard deviations away from the mean. Using Grubb's test for outliers, we determined that one data point (value = 168.3) was an outlier ($Z = 2.99$, $p < 0.05$). When this outlier was removed from the plot, there was no longer a correlation between the density of I_A and the density of $I_{K(Ca)}$. Further experiments will be necessary to determine if the lack of correlation is due to an insufficient number of data points or due to a true lack of coordinated regulation of these currents. When the densities of I_A and $I_{K(dr)}$ were

plotted, we found no correlation (Figure 3.7B), suggesting that these currents are not coordinately regulated under control conditions. No correlation was found for either I_A versus $I_{K(Ca)}$ or I_A versus $I_{K(dr)}$ after 12 hours of $GABA_A$ receptor block (Figure 3.7C). This suggests that the expression of I_A, $I_{K(Ca)}$, and $I_{K(dr)}$ are not coordinately regulated in motoneurons of the embryonic chick. However, we cannot rule out the possibility that these currents are coordinately regulated but we were unable to detect the correlation due to a relatively low sample size.

Discussion

Previously we reported that blocking $GABA_A$ receptor signaling produced a transient reduction in embryonic motor activity that homeostatically recovered to normal levels within 12 hours (Wilhelm and Wenner, 2008). We also found that reductions in GABAergic transmission triggered increases in quantal amplitude, but that these changes did not occur until after the homeostatic recovery of embryonic activity. Therefore, a separate mechanism must exist to recover embryonic activity. Here we show that reducing excitatory GABAergic transmission also triggered a homeostatic increase in cellular excitability, which occurred more rapidly than quantal changes, and therefore could explain the recovery of embryonic activity. Cellular excitability was increased through the increase in sodium currents and the reduction of fast-inactivating and sustained calcium-activated potassium currents. These changes in sodium and potassium currents are compensatory in the sense that they have been shown to be critical to increasing excitability by modulating action potential waveform and firing

frequency (McCobb et al., 1990; Sah and Bekkers, 1996; Goa and Ziskind-Conhaim, 1998; Martin-Caraballo and Greer, 2000). Together, these findings support the idea that $GABA_A$ receptor signaling is a critical part of the sensing machinery that compensates for lowered activity and triggers homeostatic changes in both cellular excitability and synaptic strength.

Reduced GABAergic transmission and network activity both trigger increased cellular excitability

Compensatory changes in cellular excitability have been shown after activity blockade invertebrate central pattern generators and mammalian cortex (Desai, 2003; Davis, 2006). Data from these diverse systems have shown that reducing activity triggers changes in cellular excitability through modification of ionic conductances. Previous studies of vertebrate central synapses have shown homeostatic increases in sodium channel currents (Desai et al., 1999a; Aptowicz et al., 2004), reductions in Ca^{2+}-activated potassium currents (Desai et al., 1999a; Martin-Caraballo and Dryer, 2002), and reductions in the density and inactivation kinetics of transient potassium channels (Casavant et al., 2004) after TTX-induced activity block. These findings are consistent with the idea that cells monitor spiking activity, and that changes in spiking activity trigger changes in cellular excitability. We have blocked $GABA_A$ transmission and see very similar results to previous studies that block activity (increases in I_{Na}, faster I_A kinetics and reductions in I_A and $I_{K(Ca)}$) suggesting a similar process has been triggered. However, in our study it is highly unlikely that reduced activity (assessed as a

reduction in limb movements) triggers these changes. Although we do see a transient reduction in spontaneous activity (SNA) following injection of the $GABA_A$ receptor antagonist, this does not appear to trigger changes in cellular excitability because block of ionotropic glutamate receptors produces the same transient reduction of activity but does not trigger any changes in cellular excitability. Our findings therefore support the idea that lowered GABAergic neurotransmission triggers compensatory changes in cellular excitability. Thus in the developing spinal cord GABA transmission appears to be sensed as a monitor of activity. In several previous studies perturbations that reduced activity also reduced the release of neurotransmitter and therefore neurotransmission could be important in triggering changes in cellular excitability in these studies as well. In fact, blockade of synaptic transmission using tetanus toxin has been shown to lead to changes in the transcriptional regulation of sodium currents (Weston and Baines, 2007). Farrant and colleagues (Brickley et al., 2001) showed that the genetic deletion of $GABA_A$ receptors in murine cerebellar granule cells triggers a homeostatic increase in voltage-independent K^+ leak channels. However, in both of the previous studies, it was not possible to separate the influence of transmission from activity. A recent study has begun to separate the contributions of activity and neurotransmission. In the crab stomatogastric ganglion, perturbation of neuromodulators, but not activity, altered the regulation of ionic conductances (Khorkova and Golowasch, 2007). Together, these findings support the role of ligand-gated channels in the sensing

machinery that triggers changes in cellular excitability in the developing spinal cord and possibly in other systems as well.

Coordinated regulation of ion channel densities

Several studies have shown activity-dependent regulation of two or more ionic currents (Lissin et al., 1998; Desai et al., 1999a; Golowasch et al., 1999). A few recent studies have suggested that multiple ionic currents can be coordinately regulated (MacLean et al., 2003; Khorkova and Golowasch, 2007). The mechanisms by which these ionic currents are regulated are unclear. In the lobster STG, I_A and the hyperpolarization-activated inward current (I_h) appear to be coordinately regulated by an activity-independent mechanism that is dependent on the transcription of the channels (MacLean et al., 2003). A second study has suggested that the STG neuromodulator proctolin can modulate the coordinated regulation of multiple ionic currents, including I_A, $I_{K(Ca)}$, and I_h (Khorkova and Golowasch, 2007). Removal of the neuromodulatory input from STG neurons caused the loss of co-regulation among these currents. Further studies are necessary to determine if $GABA_A$ neurotransmission has a similar role in coordinating the regulation of ionic currents. A loss in co-regulation after $GABA_A$ receptor block would make sense for triggering homeostatic regulation of ionic currents because that would allow for each current to be independently adjusted to create the proper balance in excitation. Together, these finding place a special importance on neurotransmission (and neuromodulation) in the process of triggering changes in the densities of ion channels.

$GABA_A$ receptor as a sensor for both cellular excitability and synaptic strength

We have now shown that reducing GABAergic transmission can trigger compensatory changes in both quantal amplitude and cellular excitability [(Wilhelm and Wenner, 2008) and this study]. Therefore this appears to be a functionally economical process as 2 compensatory mechanisms (cellular excitability and quantal strength) are both triggered via the same sensing machinery ($GABA_A$ receptor activation). Our data supports a model in which $GABA_A$ receptors sense changes in extracellular GABA as a proxy for activity, and reduced GABAergic signaling triggers increases in cellular excitability and quantal strengthening (Figure 3.8). This increase in synaptic strength could be triggered because $GABA_A$ receptors remain blocked throughout the 48-hour period, thus simulating prolonged activity blockade. As the slower responding synaptic strength begins to increase, and could contribute to increased network excitability at a time when intrinsic cellular excitability begins to return toward control levels. Although reducing activity itself may not directly trigger compensatory alterations in cellular excitability, increased activity may negatively feedback upon these compensatory changes. Forty-eight hours of $GABA_A$ blockade showed reduced cellular excitability compared to 12 hours of block even though the block was in place longer. This is consistent with the possibility that once activity recovers to control levels mechanisms can be engaged that prevent the network from overshooting normal activity levels. The importance of $GABA_A$ receptor signaling in triggering homeostatic changes in cellular

excitability and quantal strength is not surprising because $GABA_A$ signaling has been shown to be critical for several aspects of early development including cell proliferation, cell migration, cell differentiation, establishment of synaptic connections, and synaptic refinement (Ben-Ari et al., 2007).

Homeostatic changes in cellular excitability, NOT synaptic strength, lead to recovery of activity.

Blocking activity in networks of cultured neurons leads to compensatory increases in both the excitability of the postsynaptic neuron (Desai, 2003) and increases in the strength of excitatory synaptic inputs (Turrigiano, 2007). Previous studies of this kind of synaptic and cellular homeostasis have shown that compensatory changes can be observed after 24-48 hours of activity block (Desai, 2003; Davis, 2006; Rich and Wenner, 2007; Turrigiano, 2007); however see (Ibata et al., 2008). These changes in cellular excitability and synaptic strength are thought to contribute to the homeostatic recovery of normal activity levels. However most studies that block activity cannot study the process of recovery because network activity does not recover from these forms of blockade (TTX, glutamate antagonists) within the period studied. To understand the homeostatic mechanisms and their relationship to activity recovery, one would need to compare the time course of recovery and the various homeostatic mechanisms, but this has yet to be done. However, one previous study using of rat hippocampal slice in culture did find that changes in cellular excitability occur before the onset of changes in inhibitory synaptic strength (Karmarkar and

Buonomano, 2006). Some studies have observed the homeostatic recovery of normal activity levels but do not follow the timing of recovery and compare it to the onset of the compensatory mechanisms (Turrigiano et al., 1998; Burrone et al., 2002; Karmarkar and Buonomano, 2006; Pratt and Aizenman, 2007) except see (Aptowicz et al., 2004). To our knowledge the present study is the first to demonstrate that changes in cellular excitability occur during the period in which the activity is recovered and before changes in synaptic strength are engaged (Figure 3.8). This suggests the surprising possibility that cellular excitability is the mechanism that homeostatically recovers the activity, and that changes in quantal amplitude are not involved in this step of the homeostatic process. Changes in cellular excitability could be an intermediary step in triggering the slower changes in synaptic strength, which may be necessary to maintain the recovered activity levels. Alternatively, reduced $GABA_A$ receptor signaling could trigger changes in both cellular excitability and synaptic strength at the same time, but the synaptic changes could take longer to develop. In this model, changes in voltage-gated channels respond quickly and recover activity; whereas, slower responding changes in the strength of synaptic inputs serve to maintain the increase in network excitability.

Recovery of SNA after glutamatergic block.

After injection of glutamatergic receptor antagonists, embryonic movements return to control levels without triggering any sustained changes in cellular excitability or synaptic strength. Several mechanisms are likely to exist to

recover and maintain activity. Following glutamatergic blockade *in vitro*, activity quickly recovers due to a change in the loading of intracellular chloride, which strengthens the unblocked GABAergic currents (Chub and O'Donovan, 1998, 2001). This process is also likely occurring *in ovo* following glutamate blockade, thereby avoiding the need to engage compensatory mechanisms of cellular excitability and quantal strength. It is also possible that fast changes in the probability of release could be occurring (Frank et al., 2006). A final possibility is that fast, un-sustained changes in synaptic strength occur *in ovo* to facilitate the recovery after glutamatergic block, but these changes are not maintained such that quantal amplitude and/or cellular excitability return to control values during the period after isolation of the spinal cord (Kaneko et al., 2008).

Concluding remarks

Examining the time course of the different homeostatic mechanisms triggered by activity perturbations is critical to understanding the functional recovery and maintenance of activity levels. We have shown that compensatory changes in cellular excitability could act to recover perturbed activity levels. Only after network activity is recovered, do changes in quantal amplitude occur. These findings are important because they speak to the roles of different mechanisms of homeostatic plasticity play in the living embryo. It will be important for future studies to consider the time course and interaction between the multiple forms of homeostasis. Further, we show another example of the critical importance that the neurotransmitter GABA plays in early development.

Here, GABA appears to act as a proxy for activity, which is sensed through the $GABA_A$ receptor and when reduced triggers coordinated compensatory changes in I_{Na}, I_A, $I_{K(Ca)}$, GABAergic and AMPAergic quantal amplitude, and therefore could have profound effects on the maturation of cellular excitability and synaptic strength.

Experimental Procedures

Pharmacological blockade of synaptic transmission.

Embryonic limb movements were monitored as previously described (Wilhelm and Wenner, 2008). Briefly, at stage 33-34 (E8; (Hamburger and Hamilton, 1951), a window in the shell was opened to allow monitoring of chicken embryo limb movements and drug application. An aqueous solution of gabazine (or SR 95531 hydrobromide; 20 μM), 6-Cyano-7-nitroquinoxaline-2,3-dione disodium (CNQX, 20 μM), DL-2-Amino-5-phosphonopentanoic acid (APV; 100 μM), or some combination of these antagonists was injected onto the chorioallantoic membrane of the chick embryo. These concentrations were previously determined to be appropriate for blocking neurotransmission from E8-E10 (Wilhelm and Wenner, 2008). Activity was monitored by counting chick embryo limb movements during several 5-minute periods over 12 or 48 hours, until E10.

Dissection.

White Leghorn chicken embryos (Hy-Line Hatcheries, Mansfield, GA) were dissected as described previously (Wenner and O'Donovan, 2001). Briefly, spinal cords from stage 36 (E10) embryos were isolated with intact muscle nerves arising from the crural plexus. The preparation was maintained in a re-circulating bath of oxygenated Tyrode's solution and the dissection was performed at 15ºC and left to recover overnight at 17ºC. The dissection, recovery, and recording periods took place in the absence of gabazine, CNQX, and APV unless stated otherwise.

Whole cell electrophysiology: current and voltage clamp.

Whole cell voltage clamp and current clamp recordings of were obtained from E10 motoneurons in the isolated *in vitro* chick spinal cord. Whole cell current clamp recordings of rheobase currents were acquired using an AxoClamp 2B amplifier, controlled by pClamp 10.1 software (Axon Instruments) running on a laptop computer (Apple). Voltage clamp experiments to measure individual currents and current clamp recordings of firing rates were performed using an AxoPatch ID amplifier, controlled by pClamp 10.1. Series resistance and whole cell capacitance were estimated using the amplifier's built-in circuitry. Series resistance was compensated by 70-80%. Linear leak and residual capacity currents were subtracted using a P/4 subtraction protocol. Junction potentials for the various solutions were 3-7 mV and were left uncorrected. Currents were analyzed using Clampfit (Axon Instruments).

To obtain the K^+ current activation curves, values of K^+ chord conductance (G) were calculated from the respective peak currents. The K^+ equilibrium potential was determined using the Nernst equation. All activation curves were fit with the Boltzmann equation described by the following function: $y = 1/[1 + (\exp(V-V_{1/2})/\text{slope})]$, where V is the activation voltage and $V_{1/2}$ is the half-activation voltage (Brown, 2001) .

Extracellular and intracellular solutions.

All recordings were performed in oxygenated (5% $C0_2$/95% O_2) saline solution at 27ºC. For all external solutions, the osmolarity was 305-315 mOsm. The osmolarity for all intracellular solutions was 289-291 mOsm. All solutions had a pH of 7.2-7.3.

The external saline solution for recordings of rheobase currents and instantaneous firing rates contained (in mM): NaCl (139), KCl (5), $CaCl_2$ (3), $MgCl_2$ (1), $NaHCO_3$ (17), and D-glucose (12). The intracellular solution was (in mM): NaCl (10), KCl (36), K-gluconate (94), $CaCl_2$ (0.1), $MgCl_2$ (1), HEPES (10), EGTA (1.1), Na_2-ATP (1), and Mg-GTP (0.1).

The external solution for recordings of voltage-activated sodium currents contained (in mM): NaCl (139), KCl (5), $CaCl_2$ (3), $MgCl_2$ (1), $NaHCO_3$ (17), CdCl (0.4), TEA-Cl (30), 4-AP (5), D-glucose (12), gabazine (0.02), CNQX (0.02), and APV (0.05). The intracellular solution was (in mM): NaCl (10), KCl (36), K-gluconate (94), HEPES (10), Bapta (11), $MgCl_2$ (1), $CaCl_2$ (0.1), Na_2-ATP (1), Mg-GTP (0.5), TEA-Cl (10), verapamil (0.1), and CsCl (5).

For whole cell recordings of voltage-activated calcium currents, the external solution contained (in mM): NaCl (33), choline chloride (97), TEA-Cl (30), $MgCl_2$ (2), HEPES (20), $CaCl_2$ (10), 4-AP (5), D-glucose (12), TTX (0.001), gabazine (0.02), CNQX (0.02), and APV (0.05). Intracellular solution was (in mM): $CsMeSO_4$ (120), TEA-Cl (10), $MgCl_2$ (5), HEPES (10), EGTA (10), Na_2-ATP (1), and Mg-GTP (0.5). For all extracellular calcium-free solutions, $CaCl_2$ was replaced by an equimolar concentration of $MgCl_2$.

The external solution for voltage-activated potassium current experiments contained (in mM): NaCl (145), KCl (5.4), $MgCl_2$ (0.8), $CaCl_2$ (3.4), HEPES (13), D-glucose (5), TTX (0.001), gabazine (0.02), CNQX (0.02), and APV (0.05). The intracellular solution contained (in mM): KCl (120), $MgCl_2$ (2), HEPES (10), EGTA (10), Na_2-ATP (1), and Mg-GTP (0.5).

Chemicals and drugs.

Tetrodotoxin (TTX), gabazine (or SR 95531 hydrobromide), 6-Cyano-7-nitroquinoxaline-2,3-dione disodium (CNQX), and $CsMeSO_4$ were purchased from Tocris Cookson (Ellisville, MO). All other chemicals and drugs were purchased from Sigma-Aldrich (St. Louis, MO).

Statistics.

Data from averages are expressed as mean $\pm$ SEM. Significant differences were calculated using Student's unpaired t-test (α = 0.05) when single comparisons were made. Differences between multiple groups were calculated

using one-way ANOVA with post-hoc Dunnett's test (α = 0.05) or Tukey's test (α = 0.05).

Remaining Questions and Future Directions

In this chapter we showed that $GABA_A$ receptor block triggers fast increases in cellular excitability that are likely mediated by changes in the densities of inward and outward voltage-gated channels. As discussed earlier in the chapter, several studies of cultured neural networks have demonstrated similar changes in cellular excitability after activity block. However, the time-course in which changes in cellular excitability occur is unclear.

The present study is one of the first to examine the time course in which activity recovers to normal levels. Our findings that $GABA_A$ receptor block can trigger fast increases in cellular excitability within 12 hours of treatment suggest that these changes in cellular excitability could underlie the recovery of activity, which occurs within the same period. However, it is possible that other mechanisms could underlie this recovery in activity. Further studies into the onset and progression of changes in cellular excitability will be necessary to determine if the increase in cellular excitability does aid in recovery. Activity begins to recover within 2 hours after the application of a receptor antagonist (Figure 2.4). A set of experiments that measured rheobase current and spike threshold at multiple time points (such as 2, 4, and 8 hours) during the recovery could inform us about the role of cellular excitability in recovery of activity. If cellular excitability does not begin to change until 8 hours after antagonist treatment, this would suggest that an unknown mechanism, and not cellular excitability, is responsible for the onset of recovery. If changes in cellular excitability do occur along the same time as the recovery of activity, then it is

likely, but it is not proof that, these changes cause the recovery. It is likely that multiple mechanisms exist to recover normal activity levels.

Similar to $GABA_A$ receptor block, 48 hours of activity block using lidocaine triggered changes in cellular excitability (Figure 3.1 and 3.9). This change in cellular excitability is likely due to a reduction in $GABA_A$ receptor signaling because activity block reduces the presynaptic release of GABA. Examining whether 12 hours of activity block increases cellular excitability to the same degree as 12 hour of $GABA_A$ receptor block will provide more information about the similarities between activity block and $GABA_A$ receptor block. If 12 hours of activity block increases cellular excitability to the same extent as $GABA_A$ receptor block, this would suggest that the same processes are triggered by activity block and $GABA_A$ receptor block. However, activity does not recover after lidocaine treatment, so there would be no reason for cellular excitability to recover towards normal as found after 48 hours of lidocaine treatment (Figure 3.9). I predict that activity block does not increase cellular excitability to the same extent as $GABA_A$ receptor block. This would make sense as $GABA_A$ receptor signaling is not as reduced by lidocaine treatment as gabazine treatment.

Future experiments that focus on the time course over which changes in cellular excitability and quantal amplitude occur are critical to understanding how normal activity levels are recovered after activity perturbations.

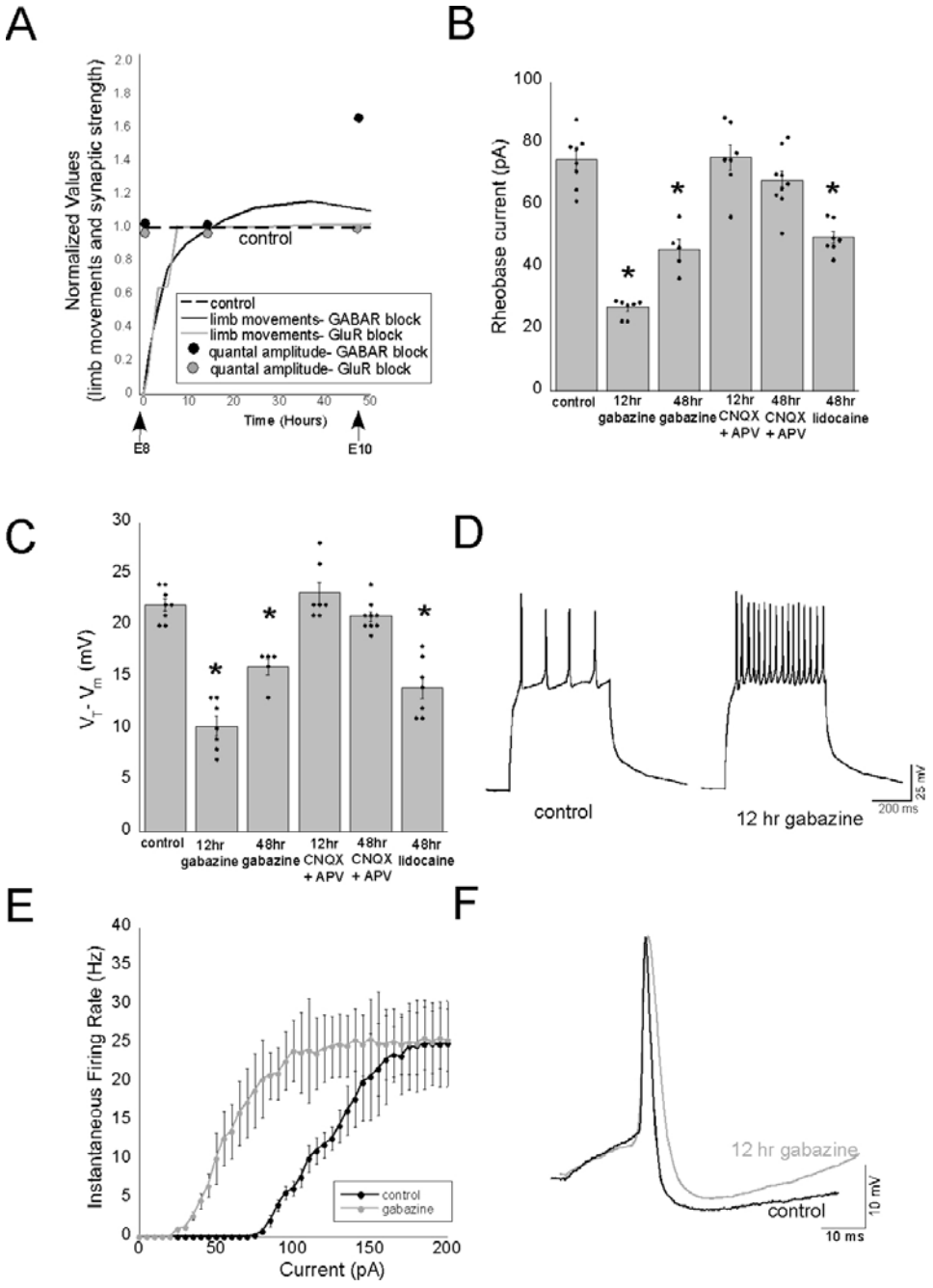

Figure 3.1. $GABA_A$ receptor block decreased spike threshold. (A) Schematic of the relationship between the recovery of SNA after neurotransmitter blockade and the subsequent increase in synaptic strength. Lines represent the average duration of limb movements (measure of SNA) for 48 hours after the injection of gabazine or CNQX + APV. Values are normalized to control (dashed

line). Blocking $GABA_A$ receptors (gabazine, solid black line) or glutamatergic receptors (CNQX + APV, solid gray line) reduces limb movements for approximately 12 hours before recovering to control levels. Closed black circles represent the quantal amplitude of excitatory GABAergic and AMPAergic currents. Excitatory synaptic strength increases within 48 hours after $GABA_A$ receptor blockade with gabazine. No change in synaptic strength is seen after 48 hours of glutamatergic receptor blockade with CNQX and APV (closed gray circles), or after 12 hours of treatment with gabazine or CNQX + APV. (B) The minimum amount of current needed to evoke a single action potential (rheobase current) was significantly reduced after activity block ($*p \leq 0.0001$) and $GABA_A$ receptor block ($*p \leq 0.0001$) but not glutamatergic block. (C) The difference between resting membrane potential (V_m) and the threshold potential (V_T) was reduced after activity block ($*p \leq 0.0001$) and $GABA_A$ receptor block ($*p \leq 0.0001$) but not glutamate receptor blockade. (D) Representative spike trains evoked by somatic current injection (110 pA) in control and gabazine-treated motoneurons. (E) Average f-I curves for control (n = 5) and gabazine-treated (n = 8) embryos. 12 hr gabazine treatment shifts the average f-I curve to the left, suggesting an increase intrinsic excitability. (F) Representative action potentials from control (black) and gabazine-treated (gray) motoneurons. 12hr gabazine treatment significantly increases spike half-width ($p \leq 0.001$) and the peak of the hyperpolarization ($p \leq 0.05$). Data in this graph and all subsequent graphs are represented as averages ± SEM.

Table 3.1. Membrane Properties

Treatment condition	n	Rheobase Current (pA)	Spike Threshold (mV)	Membrane Potential (mV)	Input Resistance (MΩ)	Membrane Capacitance (pF)
Control	8	74.6 ± 3.0	22.1 ± 0.6	-51.6 ± 0.8	384.1 ± 28.9	20.3 ± 1.5
12h gabazine	7	28.8 ± 1.1*	10.3 ± 0.9*	-50.3 ± 0.6	337.3 ± 27.2	20.5 ± 1.4
48h gabazine	5	45.8 ± 3.3*	16.0 ± 0.8*	-53.6 ± 2.6	366.2 ± 14.1	23.6 ± 7.3
12h CNQX	8	71.8 ± 3.4	20.0 ± 1.3	-50.9 ± 0.6	388.1 ± 15.0	20.3 ± 1.1
48h CNQX	8	69.8 ± 4.1	20.5 ± 0.8	-51.5 ± 0.7	329.4 ± 20.9	21.8 ± 0.5
12h CNQX + APV	7	75.5 ± 4.1	23.1 ± 1.0	-51.9 ± 0.6	383.6 ± 23.7	18.9 ± 1.4
48h CNQX + APV	9	67.9 ± 3.2	20.9 ± 0.5	-51.3 ± 0.4	377.8 ± 18.4	19.7 ± 1.2
48h lidocaine	6	49.6 ± 2.0*	14.1 ± 1.1*	-51.5 ± 0.4	327.0 ± 21.1	18.7 ± 1.7

* $p \leq 0.0001$

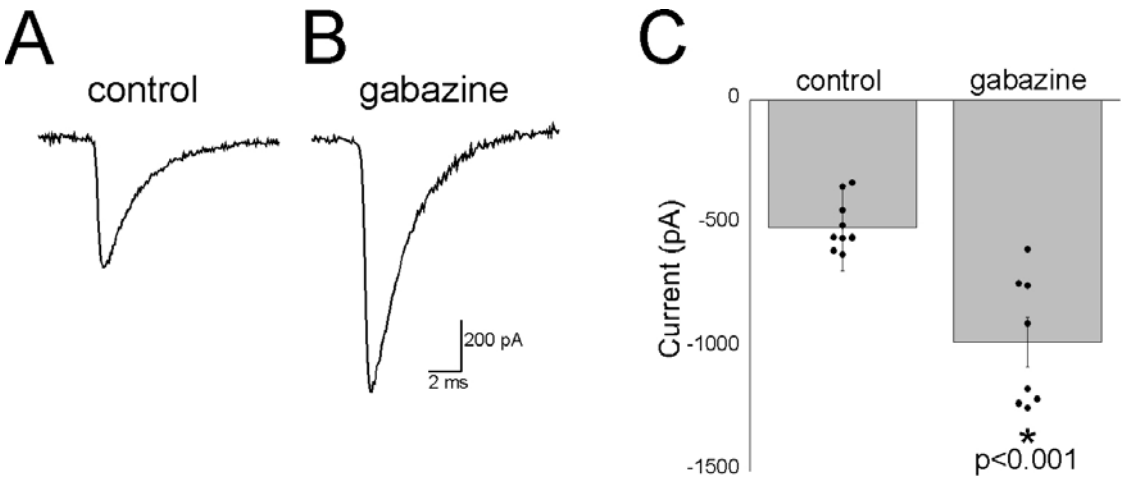

Figure 3.2. The amplitude of sodium currents was increased by 12 hours of gabazine treatment. Sample traces of sodium currents from saline- (A) and gabazine-treated (B) motoneurons elicited by a depolarizing step to -10 mV from a holding potential of -60 mV. (C) The average peak current amplitudes elicited by the voltage step to -10 mV from -60mV were significantly increased after 12 hours of $GABA_A$ receptor block (n = 8; control n = 9; $p \leq 0.001$).

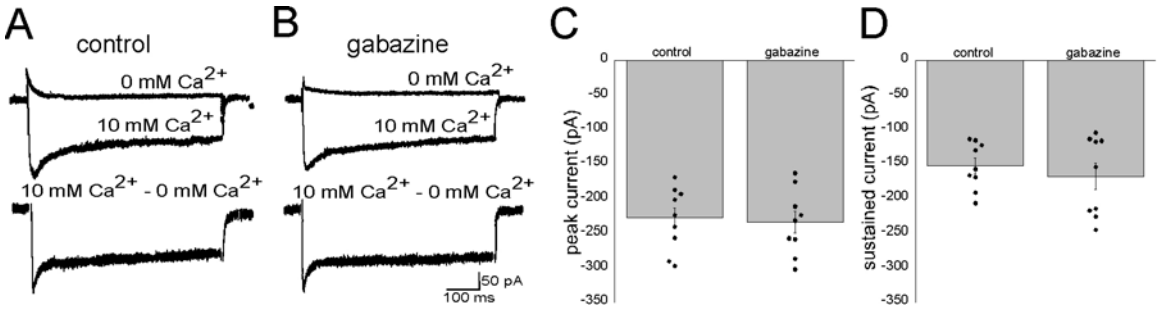

Figure 3.3. Calcium currents were not affected by 12 hours of gabazine treatment. Sample traces of control (A) and gabazine-treated (B) calcium currents elicited by depolarizing voltage steps to -10 mV from a holding potential of -60 mV in the presence and absence of external Ca^{+}(top traces). Net currents were assessed by subtracting the current elicited without calcium from current elicited with external calcium (bottom traces). The average of the peak currents elicited by the step to -10 mV from -60 mV reveal that $GABA_A$ block (n = 9) did not alter either peak (C) or sustained (D) calcium currents compared to control (n = 9).

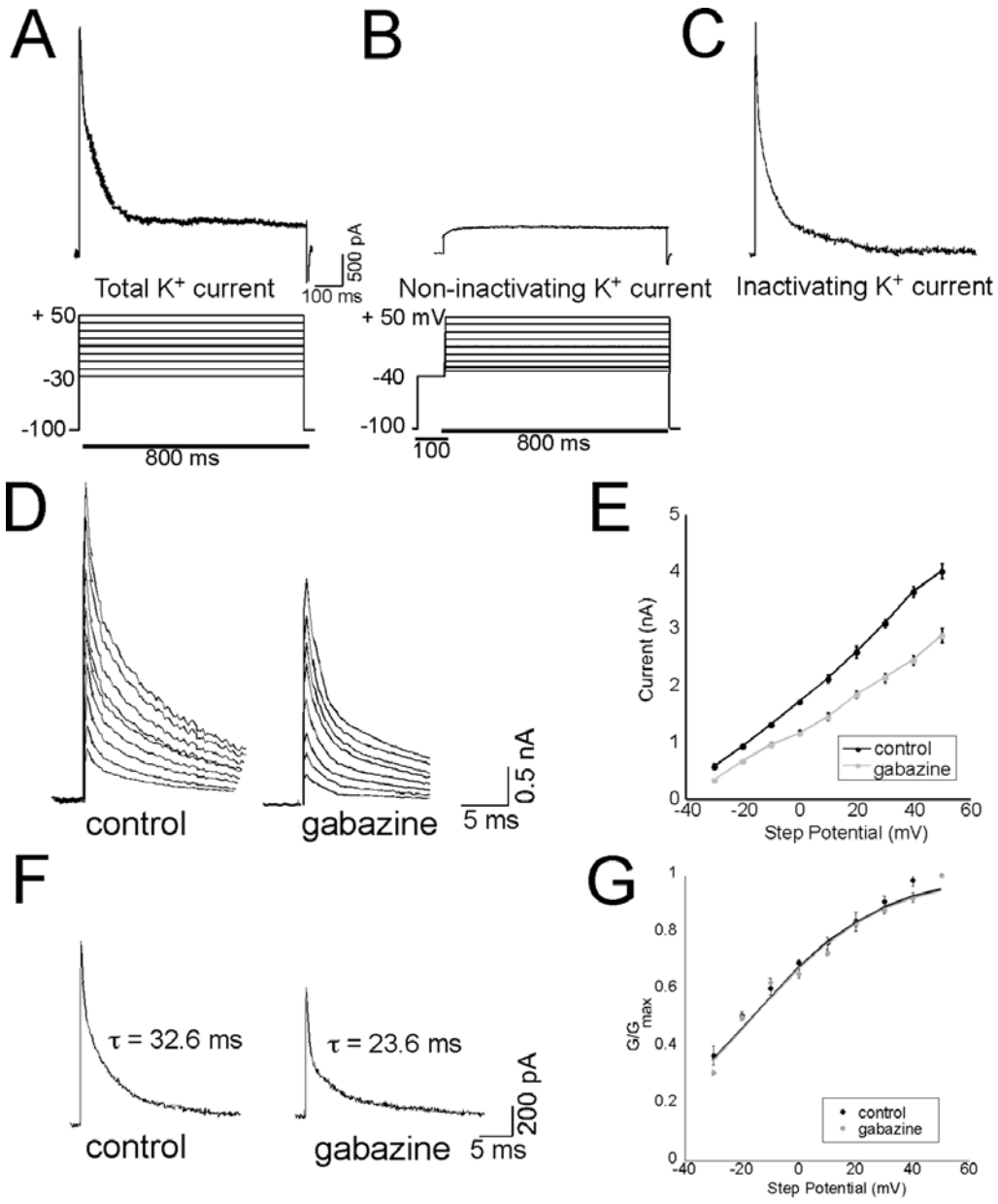

Figure 3.4. Fast inactivating potassium currents are reduced after 12 hours of gabazine treatment. (A) Representative total K^+ current elicited by a depolarizing step to +20 mV from a holding potential of -100 mV. The protocol used to evoke total K+ currents throughout a range of depolarizing steps to +50 mV from -100 mV (in 10 mV increments) is below. (B) Sustained K^+ currents (I_K) were elicited when the same series of voltage steps used in A were preceded by

a 100 ms pre-pulse to -40 mV. A representative sustained K^+ current elicited by a voltage step to +20 mV demonstrates that this current does not rapidly inactivate. (C) Difference currents (currents in B subtracted from those in A) reveal a fast-inactivating K^+ current (I_A). (D) The peak I_A currents from control (left) and gabazine-treated (right) motoneurons. (E) Gabazine treatment (n = 11) significantly reduces I_A currents compared to control (n=14). (F) Representative traces of currents evoked by voltage steps to -10 mV from control (left) and gabazine-treated (right) motoneurons with $\tau_{inactivation}$ as indicated. (G) Average conductance values normalized to the value at +50 mV produced an approximate activation curve (G/G_{max}) fitted with a Boltzmann equation. Gabazine treatment does not alter the voltage dependence of the I_A current compared to control.

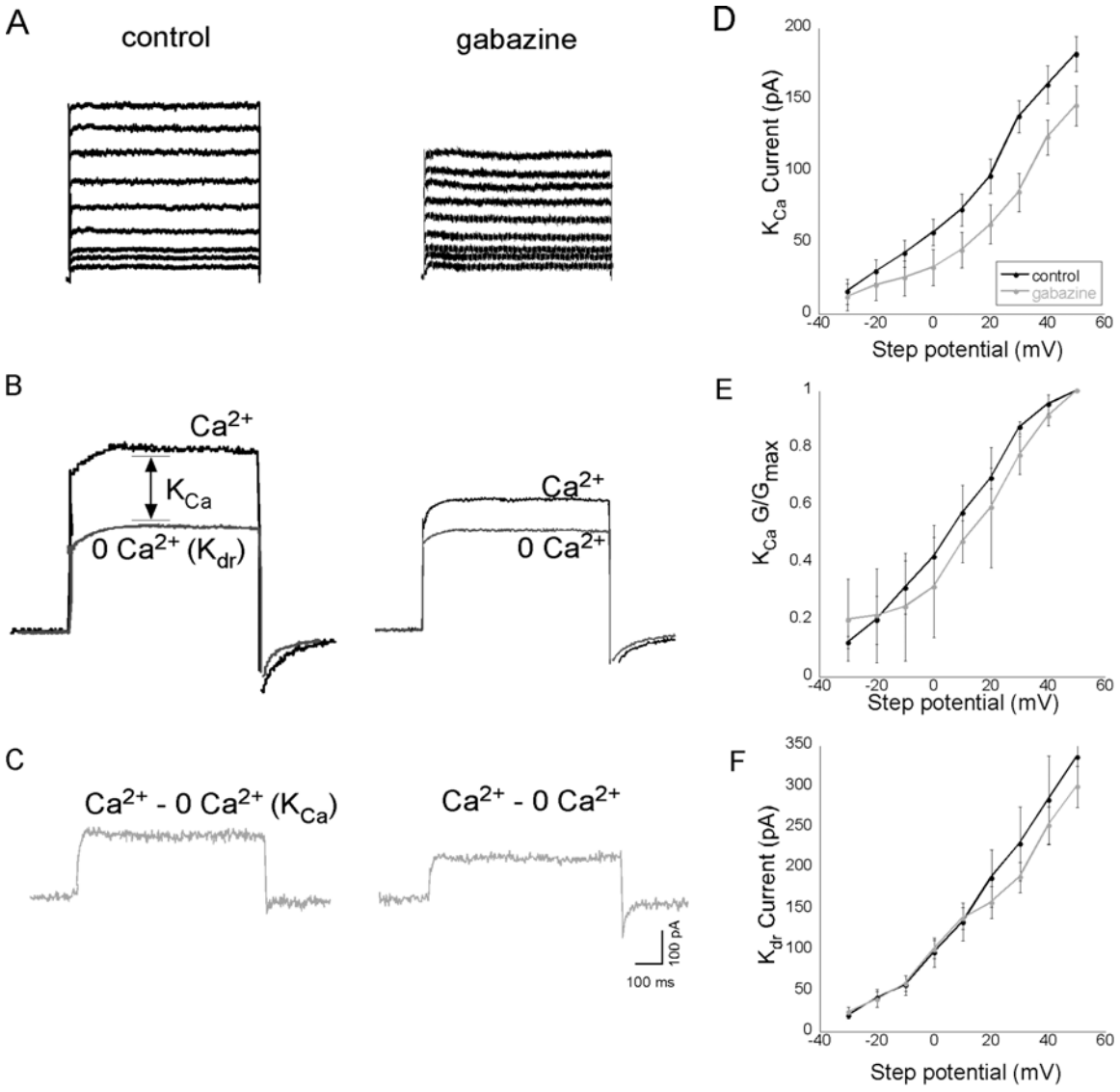

Figure 3.5. Gabazine treatment reduces calcium-activated K^+ currents but not delayed rectifying K^+ currents. (A) Representative outward currents evoked by depolarizing steps to +50 mV (in 10 mV increments) from a holding potential of -40 mV. (B) Representative currents elicited by a voltage step to +50 mV from a holding potential of -40 mV obtained in the presence (3.4 mM Ca^{2+}) and absence (0 mM Ca^{2+}) of external calcium. K_{dr} is measured as the current evoked in the absence of external calcium. (C) Net K_{Ca} currents were obtained after digital subtraction of the raw traces (Ca^2 – 0 mM Ca^{2+}).

(D) Gabazine treatment (n = 8) significantly reduced the K_{Ca} currents compared to control (n = 9). (E) No difference was found in the activation curves from gabazine-treated and control motoneurons. (F) Gabazine treatment (n = 8) did not significantly alter K_{dr} currents compared to control (n = 9).

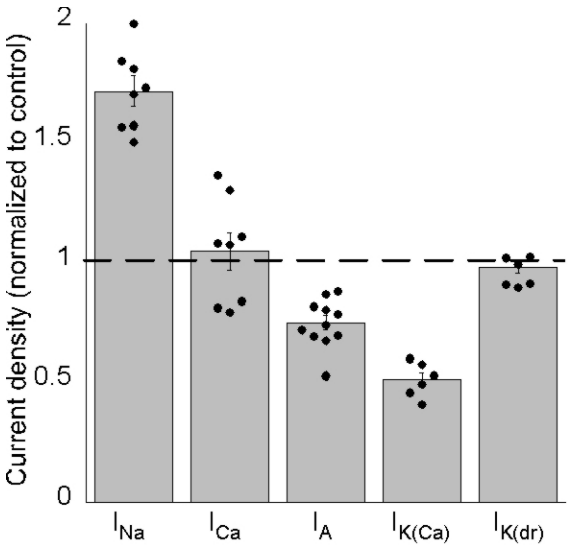

Figure 3.6. Current densities were differentially regulated by gabazine treatment. Average values for gabazine-treated neurons are plotted as a fraction of those for controls. Control is represented by dashed line. Data for I_{Na} and I_{Ca} elicited by a step to -10 mV. Data for I_A, $I_{K(Ca)}$, and $I_{K(dr)}$ were elicited by a step to +50 mV. Currents were normalized to cell capacitance.

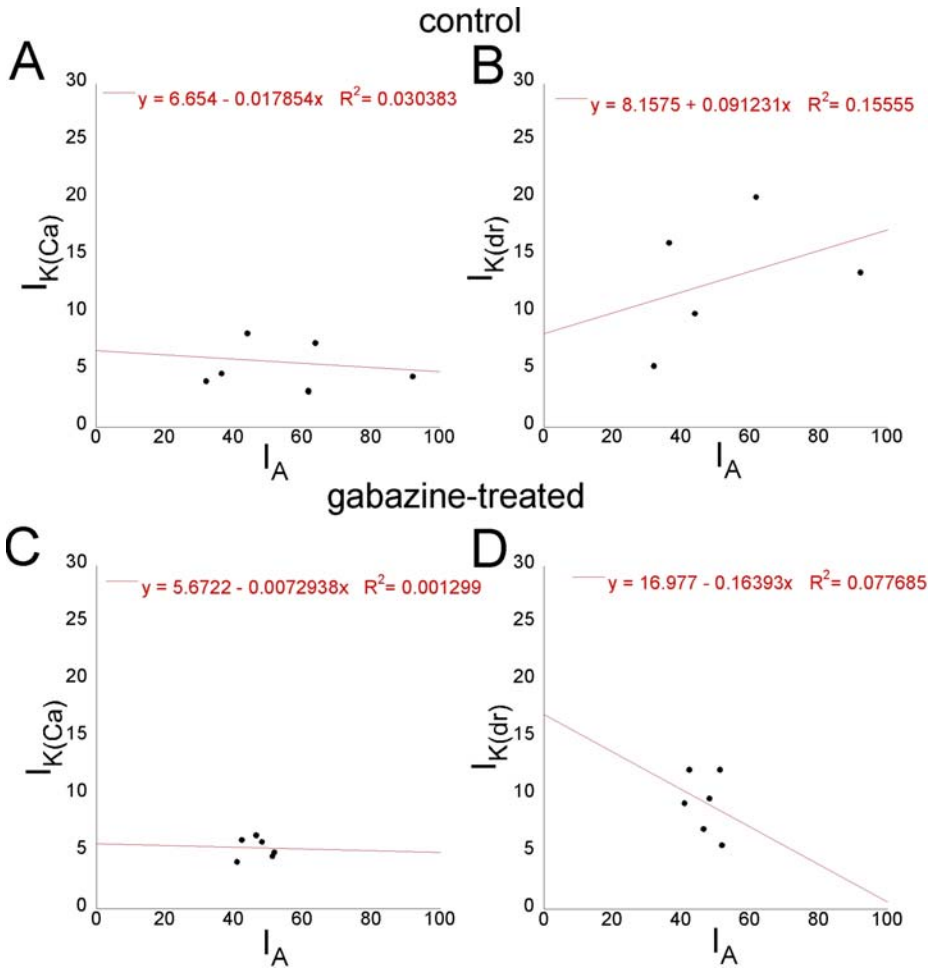

Figure 3.7. Ionic conductances do not appear to co-regulate after 12 hours of $GABA_A$ receptor block. Each point corresponds to current densities of the two indicated currents measured in an individual neuron at a step potential of +30 mV. Not all current pairs were measured in each cell, thus there are different sample sizes for different current pairs. (A) Plotting the density of I_A versus the density of $I_{K(Ca)}$ for each cell demonstrates no correlation between the density of I_A and $I_{K(Ca)}$ in control embryos. (B) $I_{K(dr)}$ current densities do not correlate with I_A current densities. (C) The densities of $I_{K(Ca)}$ and I_A do not correlate after gabazine treatment. (D) $I_{K(dr)}$ densities and I_A densities do not correlate after gabazine treatment.

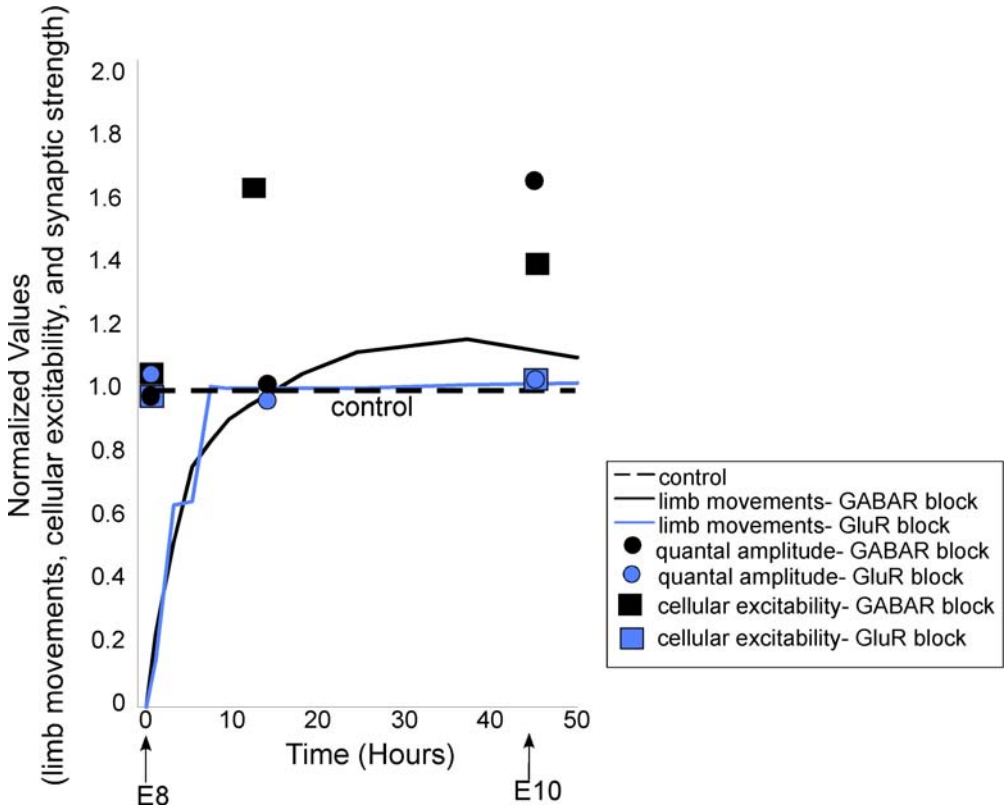

Figure 3.8. $GABA_A$ receptor block triggers a reduction in cellular excitability and an increase in quantal amplitude that could underlie the recovery and maintenance of control activity levels. The model shows the relationship between the recovery of SNA after neurotransmitter blockade and the subsequent increase in synaptic strength and reduction in cellular excitability. Lines represent the average duration of limb movements (measure of SNA) for 48 hours after the injection of gabazine or CNQX + APV. All values are normalized to control (dashed line). Blocking $GABA_A$ receptors (gabazine, solid black line) or glutamatergic receptors (CNQX + APV, solid blue line) reduces limb movements for approximately 12 hours before recovering to control levels. Closed black circles represent the quantal amplitude of excitatory GABAergic and AMPAergic currents. Excitatory synaptic strength increases within 48 hours

after $GABA_A$ receptor blockade with gabazine. No change in synaptic strength is seen after 48 hours of glutamatergic receptor blockade with CNQX + APV (closed blue circles), or after 12 hours of treatment with gabazine or CNQX + APV. Closed black squares represent neuronal excitability after $GABA_A$ receptor block; closed blue squares represent neuronal excitability after glutamatergic receptor block with CNQX + APV. $GABA_A$ receptor block reduces cellular excitability within 12 hours, and excitability begins to recover towards control levels within 48 hours of gabazine treatment. The changes in cellular excitability, but not quantal amplitude, occur at approximately the same time as the recovery of limb movements to control values after $GABA_A$ receptor block, suggesting that changes in cellular excitability underlies this recovery of activity. Changes in quantal amplitude may act to maintain activity levels once recovery has occurred.

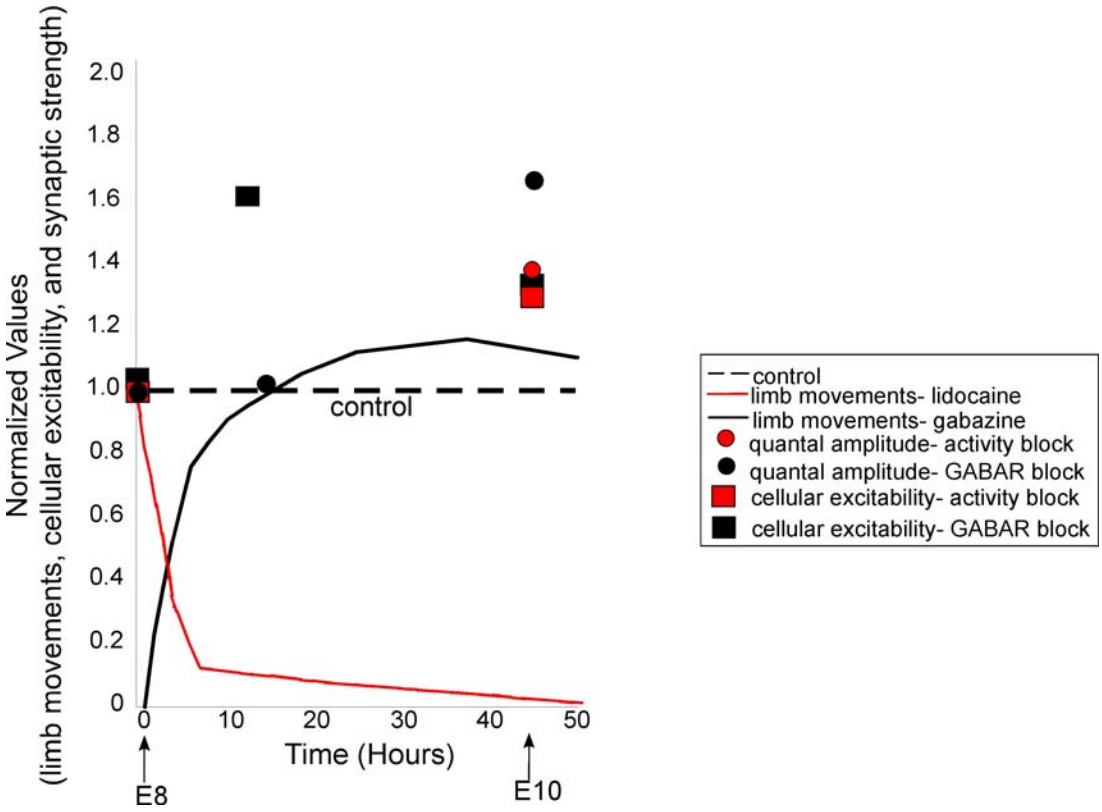

Figure 3.9. $GABA_A$ receptor block triggers changes in quantal amplitude and cellular excitability similar to those triggered after activity block. 48 hours of lidocaine treatment significantly reduces limb movements (i.e., activity; solid red line). This activity block leads to a 40% increase in the amplitude of excitatory quantal currents (closed red circles) and increases cellular excitability by 40% (closed red squares). $GABA_A$ (solid black line) transiently reduces limb movements, which recover to control levels (dashed black line) within 12 hours. Similar to activity block, $GABA_A$ receptor block triggered an increase in excitatory quantal amplitude (closed black circles) and cellular excitability (closed black squares).

CHAPTER 4:

GENERAL DISCUSSION

Previous studies have shown that activity block produced changes in both cellular excitability and synaptic strength, but the mechanisms by which the activity was being sensed by the cell or the network of cells have remained somewhat elusive. In this dissertation we demonstrate that the $GABA_A$ receptor is a critical part of the sensing machinery that triggers changes in both excitatory quantal amplitude and cellular excitability. In our model, presynaptic spiking activity causes the release of GABA, which binds to the $GABA_A$ receptor. The $GABA_A$ receptor monitors GABA binding as a proxy for activity. Therefore, reducing cell spiking activity reduces the action potential-dependent release of GABA, thereby reducing binding of GABA to the $GABA_A$ receptor. The reduction in GABA receptor activation triggers fast increases in cellular excitability by increasing the density of sodium channels and reducing the density of fast-inactivating and calcium-dependent potassium channels (Figure 3.6). This change in cellular excitability is followed by an increase in excitatory quantal amplitude (Figure 3.9). These changes in cellular excitability and quantal amplitude underlie the recovery and maintenance of normal activity levels after activity perturbations. It is unclear whether $GABA_A$ receptor block triggers changes in cellular excitability at the same time or in series with the triggering of changes in cellular excitability preceding the triggering of changes in quantal amplitude. Together, the experiments described in this dissertation are the first to identify the $GABA_A$ receptor as a molecule that is able to sense changes in activity and orchestrate changes in different ion channels as well as ionotropic signaling to compensate for the activity change.

Homeostatic regulation of cellular excitability and quantal amplitude in vitro and in vivo

Although our model allows us unparalleled access to perturbing neurotransmission *in ovo*, one disadvantage to this experimental design is that we cannot rule out the possibility that changes in cellular excitability and synaptic strength after $GABA_A$ receptor block are due to effects on descending systems as we are blocking GABAergic transmission throughout the nervous system. The fact that we are performing the study in the living embryo is also one of the significant advantages of the study.

While I have focused mainly on homeostatic processes in vertebrate central neural networks, many of the same processes have been described in invertebrate preparations, such as the crustacean STG (Turrigiano et al., 1994; Turrigiano et al., 1995) and the Drosophila neuromuscular junction (Davis and Goodman, 1998; Davis and Bezprozvanny, 2001). The presence of similar mechanisms that alter synaptic strength (both pre- and postsynaptically) and postsynaptic cell excitability in a variety of model systems suggests that there are a group of general homeostatic principles that are followed when a neuron experiences challenges to stability. While most studies of homeostatic plasticity have occurred using cultured neural networks, a few studies have demonstrated homeostatic plasticity in the intact nervous system. Monocular deprivation induced by the injection of TTX into one eye of postnatal rats triggers a process of scaling of the amplitudes of quantal currents that is similar to that

demonstrated in cultured visual cortical neurons (Desai et al., 2002). Homeostatic changes in synaptic strength and cellular excitability have also been found in neurons in central auditory nuclei (Walmsley et al., 2006). These results, together with our findings that activity and $GABA_A$ receptor block *in ovo* triggers homeostatic increases in quantal amplitude and cellular excitability, demonstrate a role for homeostatic plasticity in the intact developing nervous system.

The $GABA_A$ receptor as a critical signal during development

GABA is not a novel signaling molecule; GABA has been shown to be a critical signal in prokaryotes and eukaryotes (Ben-Ari et al., 2007). In hydra, $GABA_A$ receptors modulate the feeding response (Concas et al., 1998). In plants, GABA is important for proper development (Bouche and Fromm, 2004), guidance of pollen tubes (Palanivelu et al., 2003), and response to infection and wounded tissues (Chevrot et al., 2006). GABA has also been shown to be important for the development of beetle antennae (Wegerhoff, 1999) and the generation of mushroom bodies of the insect brain (Neckameyer and Cooper, 1998; Leal and Neckameyer, 2002). These observations suggest that GABA signaling is a highly conserved developmental signal (Ben-Ari, 2002). $GABA_A$ receptor mediated depolarization has been shown to be an important developmental cue in the developing mammalian nervous system. $GABA_A$ receptor signaling can modulate neuronal migration, act as a trophic factor to trigger proliferation and differentiation, and is involved in the establishment of

synaptic connections and synaptic refinement (Owens and Kriegstein, 2002; Kandler and Gillespie, 2005; Akerman and Cline, 2007; Ben-Ari et al., 2007). Thus, it is not surprising that $GABA_A$ receptor signaling plays a critical role in the regulation of cellular excitability and synaptic strength.

Non-specific drug effects are unlikely to cause changes in synaptic strength and cellular excitability

In order to assess the role of the neurotransmission in triggering homeostatic plasticity, we used multiple different pharmacological compounds to block ionotropic glutamatergic and GABAergic receptors. The $GABA_A$ receptor antagonists bicuculline and gabazine (also called SR95531) have been shown to be relatively selective competitive antagonists and possibly inverse agonists of the $GABA_A$ receptor (Ueno et al., 1997). However, several studies have shown that bicuculline and gabazine may also affect other ion channels and neurotransmitter systems (Seutin and Johnson, 1999). Bicuculline has been shown to block small conductance Ca^{2+}-activated potassium (SK) channels (Pflieger et al., 2002) as well as ionotropic cholinergic receptors (Zhang and Feltz, 1991). The mechanism of block of SK channels by bicuculline is unknown, but it is likely to act as a plug for the pore (Debarbieux et al., 1998). Currently there is no evidence that gabazine blocks SK channels (Seutin and Johnson, 1999). In our study, this difference between gabazine and bicuculline in blocking SK channels may account for the difference in the time course of recovery after bicuculline and gabazine treatment. The reduction in SK channels by bicuculline

could somehow prevent the induction of changes in other channels that allow for the recovery of activity. Because bicuculline produced a larger decrease in limb movements and potentially blocks SK channels, we did not use bicuculline to block $GABA_A$ receptors in studies examining the role of $GABA_A$ receptor block on changes in voltage-activated channels. Although both gabazine and bicuculline have been shown to have effects on the cholinergic system (Zhang and Feltz, 1991; Seutin and Johnson, 1999), we believe that it is unlikely that nicotinic acetylcholine receptors at the neuromuscular junction are blocked by the concentrations of $GABA_A$ receptor antagonists that we used in the described studies because embryonic limb movements occur as soon as two hours after bolus injection of the antagonist. These movements would not occur if the nicotinic receptors were blocked throughout the duration of $GABA_A$ receptor antagonist treatment. Due to the reported non-specific effects of these receptor antagonists, we decided to use either gabazine or bicuculline to block $GABA_A$ receptors. We found similar increases in quantal amplitude after $GABA_A$ receptor block regardless of whether gabazine or bicuculline was used (Figure 2.7) suggesting that the non-specific effects of either drug are not likely to have contributed to our findings.

Is the $GABA_A$ receptor a bidirectional sensor for changes in activity?

Homeostatic changes in cellular excitability and quantal amplitude have been shown to occur in a bidirectional manner. Blocking cell-spiking activity increases both cellular excitability and excitatory quantal amplitude; whereas,

increasing cell spiking activity decreases cellular excitability and excitatory quantal amplitude. We have shown that activity reductions are likely sensed through a reduction in $GABA_A$ receptor signaling. However, the question remains whether increasing excitatory $GABA_A$ signaling would trigger the same processes that are triggered by increasing cell spiking activity. Is the $GABA_A$ receptor involved in sensing both increases and decreases in activity? To answer this question, one would want to increase $GABA_A$ receptor signaling without altering cell spiking activity. A few studies have increased $GABA_A$ transmission by treating developing chick embryos *in ovo* with the $GABA_A$ receptor agonist muscimol. However, muscimol treatment virtually abolishes all spontaneous embryonic limb movements (Martin-Caraballo and Dryer, 2002). This cessation in limb movements is likely due to a depletion of intracellular chloride that reduces the chloride driving force and therefore prevents the generation of episodes of SNA. Interestingly, the increase of $GABA_A$ receptor signaling using muscimol leads to decreases in the densities of potassium currents (and likely increases in cellular excitability) similar to those reported after activity block using TTX (Martin-Caraballo and Dryer, 2002; Casavant et al., 2004). This finding, again, is consistent with the idea that increased $GABA_A$ receptor activation depletes intracellular chloride, therefore reducing the ability of the cell to depolarize. In this model, increasing $GABA_A$ receptor activation could produce some of the same effects as blocking membrane depolarization or cell spiking activity using TTX. As muscimol is six-fold more potent at the $GABA_C$ receptor than the $GABA_A$ receptor (Woodward et al., 1993), it is possible that muscimol

triggers different effects by activating the $GABA_C$ receptor in addition to the $GABA_A$ receptor. Thus, rather than using muscimol to increase $GABA_A$ receptor signaling, one could use a benzodiazepine, which increases the efficacy of the $GABA_A$ receptor opening in response to GABA binding. Recent studies in the Wenner lab have suggested that low concentrations of a benzodiazepine can be injected onto the chorioallantoic membrane of a developing chick for 48 hours without substantially altering the frequency of SNA *in ovo* (Peter Wenner, personal communication). Previous studies have suggested that the mechanisms engaged in recovering activity after increases in cell spiking are different than those engaged after activity block (Rutherford et al., 1998). It will be important to determine if the same sensors are used to detect increases and decreases in activity.

Potential signaling pathways initiated by $GABA_A$ receptor block

To implement homeostatic plasticity, a cell or network of cells must be able to sense some aspect of network activity and be able to integrate that signal over time such that large perturbations to activity can be compensated to return activity within a set range. Our findings suggest that the $GABA_A$ receptor is part of the machinery that senses changes in network activity, but the mechanisms by which the reduction in $GABA_A$ receptor signaling is translated into changes in cellular excitability and quantal amplitude are unclear. In fact, the location of the $GABA_A$ receptors involved in triggering changes in quantal amplitude and cellular excitability are unknown. The receptors could be located presynaptically,

postsynaptically, or on adjacent glia. Regardless of the location of the receptors, several molecules are likely to be involved in triggering changes in cellular excitability and quantal amplitude (Figure 1.4). In addition, different molecules may be involved in producing the different changes. I will discuss two possible signaling molecules that could be part of the downstream pathways initiated by reduced $GABA_A$ receptor signaling. These signaling pathways are speculative at this time, and many further experiments are necessary to determine if the described molecules are involved in the process of homeostatic plasticity triggered after $GABA_A$ receptor block.

Intracellular calcium signaling has been shown to be important in many types of plasticity, including LTP and LTD; thus, it is likely that calcium signaling is involved in the processes of triggering homeostatic changes in synaptic strength and cellular excitability after activity perturbations. There have been multiple physiological and computational studies that have demonstrated a role for calcium signaling in triggering changes in cellular excitability and activity-dependent ionic conductances (Liu et al., 1998; Zhang and Linden, 2003); however, there is less data on the role of calcium in homeostatic changes in quantal amplitude (Turrigiano, 2007). Calcium signals can be provided by influx through voltage-gated calcium channels or by mobilization from internal stores. Reductions in cell spiking activity or reduced depolarization after $GABA_A$ receptor block can reduce intracellular calcium concentrations by altering both sources of calcium. Blocking cell-spiking activity will prevent the flux of calcium through voltage-sensitive calcium channels as well as calcium-permeable AMPA and

NMDA receptors. The reduction in released neurotransmitter could also reduce activation of G-protein coupled neurotransmitter receptors that can trigger the release of calcium from intracellular stores. The reduction in intracellular calcium can be sensed through several calcium-sensing molecules (reviewed in (Zhang and Linden, 2003)). Calcium binding to calmodulin can directly activate multiple kinases and phosphatases, including phosphokinase C and calcium/calmodulin-dependent protein kinase II (CAMKII). Calcium can also indirectly activate cascades that involve adenylyl cyclase/cAMP/protein kinase A and nitric oxide synthase/guanylyl cyclase/cGMP/cGMP-dependent protein kinase. These multiple pathways activated by intracellular calcium can alter intrinsic excitability through directly phosphorylating voltage-gated ion channels or through phosphorylating associated proteins (Schorge et al., 1999; Morozov et al., 2003; Pittenger and Kandel, 2003). The mechanism by which changes in calcium concentrations could alter quantal amplitude is less clear. However, one study has shown that inhibition of the calcium/calmodulin-dependent family of kinases (KN93) prevents activity-deprivation induced increases in quantal amplitude, suggesting that calcium could trigger changes in synaptic strength through the activation of calcium-dependent kinases (Thiagarajan et al., 2002).

BDNF signaling has been implicated in the homeostatic regulation of both cellular excitability and synaptic strength. Previous studies have shown that the production of BDNF (Isackson et al., 1991; Zafra et al., 1991; Castren et al., 1992; Ghosh et al., 1994) and the release of BDNF (Wetmore et al., 1994; Blochl and Thoenen, 1995) are regulated by activity. BDNF is normally released by

activity and constant levels of BDNF are thought to help maintain normal activity levels (Rutherford et al., 1998). Blocking BDNF binding to the TrkB receptor triggers increases in excitatory quantal amplitude (Rutherford et al., 1998) and cellular excitability (Desai et al., 1999b). These findings suggest that BDNF is part of the machinery that produces changes in cellular excitability and synaptic strength in response to activity block. Studies in cultured networks have found that $GABA_A$ receptor signaling can induce the release of BDNF (Obrietan et al., 2002). Therefore, if activity promotes the release of BDNF via the activation of $GABA_A$ receptors, then blocking $GABA_A$ receptors would reduce the release of BDNF. The decrease in BDNF binding to TrkB receptors could then trigger increases in cellular excitability and quantal amplitude. The mechanisms by which BDNF signaling alters cellular excitability and quantal amplitude are not understood. BDNF dependent activation of the TrkB receptors can alter several different pathways including MAP kinase, protein kinase A, and phospholipase C (Obrietan et al., 2002). These downstream pathways could influence ionic channels or ionotropic neurotransmitter receptors. The time course over which BDNF signaling produces the changes in cellular excitability and quantal amplitude are not understood either. Currently studies have only examined changes in these parameters after 48 hours of activity block (Rutherford et al., 1998; Desai et al., 1999b). Future studies into whether BDNF signaling is involved in triggering changes in cellular excitability and quantal amplitude after $GABA_A$ receptor block as well as the time course over which BDNF signaling can

act are critical to understanding the role that BDNF plays in homeostatic processes.

Other sensors are necessary for the homeostatic regulation of activity

In addition to the $GABA_A$ receptor, multiple other sensors must exist to homeostatically recover and maintain normal activity levels. Our data suggest that at least one other sensor is present because continued $GABA_A$ receptor block does not continue to increase, but actually begins to decrease, cellular excitability. Therefore a second sensor that monitors a different aspect of activity must be involved in the recovery and maintenance of normal activity levels. If $GABA_A$ receptors were the only sensor that translated changes in activity into changes in cellular excitability, then 48 hours of continual $GABA_A$ receptor block should produce the same or larger increase in cellular excitability than is produced in 12 hours of $GABA_A$ receptor block. Whereas, we find that after 48 hours of $GABA_A$ receptor block, cellular excitability is beginning to return to control levels. This finding heavily suggests the presence of a second sensor.

After 12 hours of $GABA_A$ receptor block, activity levels return to control levels. This recovery of activity could trigger a new set of homeostatic mechanisms that prevents activity levels from increasing. If this second sensor did not exist then the continued $GABA_A$ receptor block could cause the continued increase in cellular excitability, which might increase activity levels above normal levels. A second set of sensors that detect increases in cell spiking activity could

provide negative feedback onto the mechanisms that control cellular excitability to keep activity levels under the upper limit.

It would also make sense that there is a sensor that monitors synaptic strength and cellular excitability. This sensor could monitor synaptic strength and provide feedback onto cellular excitability such that once increases in quantal amplitude begin cellular excitability begins to decrease. This type of feedback system could be important for regulating the balance between changes in cellular excitability and synaptic strength.

Interestingly, activity recovery occurs prior to changes in quantal amplitude. The recovery of normal activity levels by changes in cellular excitability should preclude the need for triggering a homeostatic increase in quantal amplitude. Further studies into the time course in which the changes in cellular excitability and quantal amplitude occur are necessary to determine the role that each play in recovering and maintaining normal activity levels. Several possibilities exist to explain why both cellular excitability and quantal amplitude are altered.

Changes in cellular excitability may be engaged to recover activity; whereas, changes in quantal amplitude may be engaged to maintain activity. Fast changes in cellular excitability may not be sustainable, thus, necessitating a change in quantal amplitude. In this model, a sustained increase in excitability could trigger changes in synaptic strength that would negatively feedback onto cellular excitability. Having a negative feedback between cellular excitability and

quantal amplitude could provide a powerful method for ensuring that the homeostatic processes do not increase activity levels beyond certain limits.

Different aspects of activity that are currently underappreciated could be differentially regulated by cellular excitability and quantal amplitude. The question still remains as to which features of activity are being homeostatically maintained. I have discussed the homeostatic regulation of firing rates; however, it is likely that other things are homeostatically regulated as well. In the crustacean STG activity-dependent changes in ionic conductances occur to regulate the pattern of firing rather than simply the rate of firing (Turrigiano et al., 1994). This homeostatic regulation of firing pattern may also occur in organotypic rat spinal cultures (Galante et al., 2001).

Is neurotransmission part of the sensing machinery in mature neurons?

It is unknown if the same sensors are used to monitor changes in activity during development and in maturity. The response of a neuron to changes in activity appears to be different during development than in maturity. Blocking activity in developing neurons primarily triggers increases in quantal amplitude; whereas, blocking activity in more mature neural networks trigger changes in both pre- and postsynaptic responses (Murthy et al., 2001; Burrone et al., 2002; Wierenga et al., 2006). These findings suggest that different sensors may be in place in the mature system. Several studies have demonstrated that neurotransmission may remain a part of the sensing machinery in mature systems, although the main neurotransmitter responsible for triggering changes

in excitatory synaptic strength could be glutamate rather than GABA, which is hyperpolarizing and inhibitory in the mature system (Burrone et al., 2002; Stellwagen et al., 2005). Malenka and colleagues (Stellwagen et al., 2005) have suggested that glia monitor extracellular glutamate concentrations to track activity levels. Decreases in glutamate can trigger the release of TNF-alpha from glia which leads to the homeostatic accumulation of postsynaptic AMPA receptors. GABA may continue to play a role in sensing changes in activity, but may sense increases in activity rather than decreases. Several studies have shown that blocking $GABA_A$ receptors in mature networks triggers a process that homeostatically decreases excitatory quantal amplitude. However, in these studies activity is increased because blocking $GABA_A$ receptor signaling blocks the majority of inhibitory inputs. Further studies are necessary to fully appreciate the role of neurotransmission as part of the sensing machinery in mature systems.

Concluding remarks

Generating and maintaining stability is a critical problem for developing neural networks. Changing strength of a specific group of synapses or the excitability of individual cells is not important if these properties do not work together to produce stable network function. Hebbian processes which occur over a short time scale allow for the modification of synaptic weights in a synapse specific manner but can produce unstable firing rates. By stabilizing network excitability, homeostatic plasticity is likely to be an important partner/complement

to Hebbian plasticity to allow for the development of functional networks. This type of plasticity is important because it provides a way to control the total amount of excitation or inhibition a neuron receives, while preserving any relative differences in synaptic weights.

Many important questions still remain for the field to address, including how many different ways is activity sensed, the molecular identity of the mechanisms that integrate activity over time, and whether homeostasis occurs in a cell autonomous manner or is network-dependent. This thesis has helped to partly answer the question of how changes in activity are sensed and translated into homeostatic changes in cellular excitability and synaptic strength. The data in this thesis shows that the $GABA_A$ receptor is a critical part of the machinery that senses changes in activity and triggers homeostatic increases in both quantal amplitude and cellular excitability. In addition, we have outlined a mechanism by which the changes in cellular excitability could occur (changes in ionic current densities). While I have limited my focus of homeostatic plasticity to changes in quantal amplitude, frequency of quantal currents, and intrinsic excitability, there are likely many more mechanisms that engaged to homeostatically regulate activity. It will be important for future studies to examine the time course of recovery of activity to gain a better appreciation for the roles of different homeostatic processes engaged after activity perturbations.

Understanding the mechanisms that drive homeostatic control of synaptic strength and cellular excitability provides insight into the impact of perturbations that can occur during development. If pregnant women are prescribed

medications that reduce fetal kicking activity (SNA) or block molecules that are important in mediating homeostatic processes, errors in the proper development of networks could occur. If network excitability is altered during a critical stage in development and the perturbations cannot be compensated for by the appropriate homeostatic mechanisms, then serious developmental problems could occur. Improving our understanding of the homeostatic mechanisms that are engaged after chronic activity perturbations, could allow us to provide more personalized care for pregnant women and infants.

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