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## Rab3A as a Modulator of Homeostatic Synaptic Plasticity

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# **RAB3A AS A MODULATOR OF HOMEOSTATIC SYNAPTIC PLASTICITY**

A dissertation submitted in partial fulfillment of the  
requirements for the degree of  
Doctor of Philosophy

By

ANDREW G. KOESTERS  
B.A., Miami University, 2004

2014  
Wright State University

WRIGHT STATE UNIVERSITY  
GRADUATE SCHOOL

August 22, 2014

I HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER MY SUPERVISION BY Andrew G. Koesters ENTITLED Rab3A as a Modulator of Homeostatic Synaptic Plasticity BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Doctor of Philosophy.

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## ABSTRACT

Koesters, Andrew G. Ph.D. Biomedical Sciences PhD Program, Wright State University, 2014. Rab3A as a Modulator of Homeostatic Synaptic Plasticity.

The nervous system is faced with perturbations in activity levels throughout development and in disease or injury states. Neurons need to adapt to these changes in activity, but also need to maintain circuit firing within a normal range to stabilize the network from becoming too excited or too depressed. Homeostatic synaptic plasticity, the compensatory increase or decrease in synaptic strength as a result of excessive circuit inhibition or excitation, is a mechanism that the nervous system utilizes to keep network activity at normal levels. Despite intense effort, little is known about the mechanisms underlying homeostatic synaptic plasticity. Numerous studies have implicated postsynaptic modulation of AMPA receptors, but disagreement exists as to which receptor subtype, GluR1 or GluR2, predominates. Here, we demonstrate the completely novel finding that a presynaptic protein, Rab3A, a small GTPase that binds synaptic vesicles by switching between its active GTP-bound form and its inactive GDP-bound form, is essential for the regulation of homeostatic synaptic plasticity in dissociated mouse cortical neuron cultures. Using a combination of electrophysiology, pharmacology, and immunohistochemistry, we show that multiple mechanisms exist to increase

synaptic strength in response to chronic activity deprivation, including but not limited to modulation of GluR1 and GluR2-containing AMPA receptors. Despite the variability and complexity of underlying mechanisms mediating the change in synaptic strength, we consistently found that modulation of synaptic strength in response to chronic network activity deprivation was completely lost in the absence of neuronal Rab3A, and that loss of Rab3A prevented the homeostatic increase in GluR2 levels but not GluR1. We conclude that there exist a biphasic mechanism for homeostatic synaptic plasticity, as suggested for LTP, where the induction (Phase 1) of the homeostatic increase in synaptic strength is first due to increasing GluR1-containing AMPA receptors, which is then followed by a Rab3A-dependent switch to GluR2-containing AMPA receptors to maintain the increase in synaptic strength (Phase 2).

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## **I. INTRODUCTION**

### **Synaptic Plasticity**

The nervous system is continuously confronted with altering states of activity. Numerous studies over many years have demonstrated that the nervous system is exceptionally proficient in responding to varying degrees of stimuli to remodel its circuitry in order to respond and adapt to the dynamic stream of synaptic activity. This process of activity-dependent modifications in synaptic physiology is called synaptic plasticity. Effective functioning of this process underlies normal nervous system functions such as movement, thinking, learning and memory, and perception, but malfunction of this system could trigger a variety of psychiatric and neurological disorders, possibly resulting in epilepsy, schizophrenia, or Alzheimer's disease.

Synaptic plasticity occurs in at least two forms that have opposing effects on synaptic physiology. The first is Hebbian plasticity, which includes long-term potentiation (LTP) and long-term depression (LTD), and induces rapid, long-lasting changes at individual synapses in response to altered activity. LTP is induced by high frequency bursts of activity that strengthen synapses and LTD is induced by persistent, low frequency activity to weaken synapses. This form of synaptic plasticity is believed to be the basis for learning and memory. If left unchecked LTP and LTD may promote network instability because as synaptic

strength increases unchecked at individual synapses runaway excitation could occur, and unregulated depression at single synapses may cause those synapses to drop out of the network. The second form is homeostatic synaptic plasticity, the nervous system's response to long-lasting, chronic perturbations in synaptic activity. This type of plasticity is induced by chronic perturbations to network activity and affects synaptic networks globally to maintain synaptic activity within a desired range in a compensatory manner to guard against network hyperexcitation and silencing.

### **Hebbian Plasticity**

Donald Hebb first proposed his theory on the function of neurons and psychological processes, such as learning and memory, in 1949. This later became the basis for Hebbian synaptic plasticity, which more specifically includes LTP and LTD.

Long-term potentiation is the persistent increase in synaptic strength of an excitatory postsynaptic neuron as a result of high-frequency stimulation of a synaptically connected presynaptic neuron. Induction of LTP requires the activation of *N*-methyl-D-aspartate (NMDA) receptors (Bliss and Collingridge, 1993) via postsynaptic depolarization due to cation influx through  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Malenka and Nicoll, 1999). AMPA receptors are ionotropic and are responsible for the majority of excitatory neurotransmission in the brain. NMDA receptors are also ionotropic but are blocked by  $Mg^{2+}$  at resting membrane potentials until sufficient

postsynaptic depolarization removes the block allowing  $\text{Ca}^{2+}$  influx. Activation of NMDA receptors and the subsequent influx of  $\text{Ca}^{2+}$  into the postsynaptic neuron results in an increase in the dendritic spine  $\text{Ca}^{2+}$  concentration triggering second messenger activation and a signaling cascade to recruit more AMPA receptors into the postsynaptic membrane.  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II (CamKII) has been implicated as a major player in NMDAR-dependent LTP induction (Lisman et al., 2002; Malenka and Nicoll, 1999). Other potential signaling molecules involved in LTP include other kinases, such as protein kinase A (PKA) (Lynch, 2004), protein kinase C (PKC) (Bliss and Collingridge, 1993; Hu et al., 1987; Linden and Routtenberg, 1989; Malenka and Nicoll, 1999; Malinow et al., 1989), protein kinase M zeta (PKM $\zeta$ ) (Hrabetova and Sacktor, 1996; Ling et al., 2002), proto-oncogene tyrosine-protein kinase (Src) (Salter and Kalia, 2004), mitogen-activated protein kinase (MAPK), which activates extracellular signal-related kinases (ERKs) (Sweatt, 2004; Thomas and Huganir, 2004), and phosphatidylinositol 3-kinase (PI3 kinase), an important molecule for trafficking of AMPA receptors to hippocampal synapses in dissociated cultures (Man et al., 2003). The trafficking of AMPA receptors to the synapse has been clearly demonstrated to be important for the expression and maintenance of LTP (Malenka and Bear, 2004; Malinow and Malenka, 2002; Shi et al., 2001).

The expression of LTP has been the focus of intense research and initially there was intense debate over whether it was due to a pre- or postsynaptic mechanism. Presynaptic mechanisms would involve changes affecting the release of neurotransmitter, such as increases in vesicle loading of transmitter

and increases in the probability of release (Ho and Shen, 2011; Malinow and Tsien, 1990). Postsynaptic mechanisms would involve synaptic modifications to postsynaptic receptors, such as increased insertion of AMPA receptors to enhance responsiveness to a stimulus. Ultimately, evidence accumulated that the mechanism of increasing AMPAR numbers in response to activity-dependent changes is the major mechanism in the expression of LTP (Bredt and Nicoll, 2003; Ho and Shen, 2011; Malenka and Nicoll, 1999; Malinow and Malenka, 2002; Song and Huganir, 2002). These changes in AMPA receptor - heterotetramers composed of four subunits, GluR1-4 (Greger et al., 2007) - expression appears to be due to phosphorylation of these receptors (Malenka and Bear, 2004), specifically the GluR1 subunit and its phosphorylation by CamKII, which triggers the insertion of receptors in the synaptic membrane (Hayashi et al., 2000; Lee et al., 2003).

Late-phase maintenance of LTP requires protein synthesis and gene transcription (Abraham and Williams, 2003; Lynch, 2004; Pittenger and Kandel, 2003), but another theory is that synapses that have undergone LTP exhibit anatomical remodeling to preserve the changes in synaptic strength (Malenka and Bear, 2004). These changes include growth of new dendritic spines, increases in the size of existing spines, and the splitting of spines into two functional synapses (Abraham and Williams, 2003; Yuste, 2011). Molecular mechanisms affecting these structural changes have received considerable attention and when postsynaptic scaffolding proteins are overexpressed in dissociated cultures, there is an increase in dendritic spine size (Hering and

Sheng, 2001). This has led to a model of LTP where the induction involves insertion of GluR1 AMPA receptors followed by the accumulation of scaffolding proteins into the postsynaptic density (PSD) resulting in an increase in spine and PSD size (Lisman and Zhabotinsky, 2001; Luscher et al., 2000). This model suggests that LTP first involves an increase in synaptic strength followed by growth of the synapse.

Long-term depression (LTD) is the long-lasting decrease in synaptic strength due to low-frequency stimulation of paired excitatory neurons. As with LTP, LTD induction requires both AMPA and NMDA receptor activation (Dudek and Bear, 1992; Mulkey and Malenka, 1992) and the subsequent increase in NMDAR-mediated  $\text{Ca}^{2+}$  influx. However, unlike LTP where the rise in  $[\text{Ca}^{2+}]$  is large and sudden,  $\text{Ca}^{2+}$  entry to trigger LTD is slower and less drastic (Mulkey and Malenka, 1992). This slow, persistent rise in  $[\text{Ca}^{2+}]$  activates serine-threonine protein phosphatases that de-phosphorylate AMPA receptors triggering their removal from the cell surface (Mulkey et al., 1994; Mulkey et al., 1993) (Mulkey and Malenka, 1992). Induction of LTD is blocked by phosphatase inhibitors that target calcineurin and protein phosphatase 1 (PP1) in the postsynaptic cell (Kirkwood and Bear, 1994; Mulkey et al., 1994; Mulkey et al., 1993). As in LTP, LTD expression and maintenance are reliant on AMPA receptors. De-phosphorylation of AMPA receptors, specifically the GluR1-containing subtype, decreases channel open probability (Banke et al., 2000), and also underlies the loss of AMPA receptors at synapses by internalizing them via a clathrin-mediated process (Beattie et al., 2000). Postsynaptic density protein

95 (PSD-95), a protein involved in the docking of AMPA receptors in the postsynaptic membrane, is directly bound to the postsynaptic membrane by palmitoylation and also binds stargazin, a protein demonstrated to traffic AMPA receptors to synapses (Chen et al., 2000; Schnell et al., 2002). As evidenced by the overexpression and removal of PSD-95 from the synapse, AMPAR numbers increase and decrease, respectively (Schnell et al., 2002).

Long-term potentiation and long-term depression are widely believed to be the major cellular mechanism for learning and memory by rapidly fine-tuning individual synapses based on their experiences. This constant strengthening and weakening of synapses has the potential, if left unregulated, to generate an unstable network.

### **Homeostatic Synaptic Plasticity**

Homeostatic synaptic plasticity, a negative feedback mechanism, was first described in 1998 as a global compensatory change in synaptic strength resulting from chronic perturbations to neuronal network activity (O'Brien et al., 1998; Turrigiano et al., 1998) and is proposed to protect against network instability that may be produced by LTP and LTD (Pozo and Goda, 2010). When the Na<sup>+</sup> channel blocker, tetrodotoxin (TTX), was applied to rat cortical neuron cultures for 48 hours to block all action potentials and chronically silence all synaptic activity, there was an enhancement in synaptic strength as evidenced by the increase in the amplitude of the postsynaptic response to a single vesicle release of neurotransmitter - miniature excitatory postsynaptic currents

(mEPSCs) - of pyramidal neurons (Turrigiano et al., 1998). The converse effect was observed on mEPSC amplitude when bicuculline, a GABA<sub>A</sub> receptor antagonist, was applied to cultures for 48 hours to block all inhibitory transmission to cause global network hyperexcitation resulting in decreased mEPSC amplitude (Turrigiano et al., 1998). The same compensatory effects were seen on cultured spinal neuron mEPSC amplitudes when AMPA receptor antagonists (to block all excitatory neurotransmission) or GABA<sub>A</sub> and kainate receptor antagonists (to increase basal excitatory synaptic activity) were chronically applied to cultures, and these changes in mEPSC amplitude were accompanied with an accumulation or decrease in synaptic AMPA receptors, respectively (O'Brien et al., 1998). Unlike many forms of synaptic plasticity that require Ca<sup>2+</sup> influx via postsynaptic NMDA receptors (Linden and Connor, 1995), the change in synaptic strength was, at least, in part due to AMPA receptor signaling, because cultures grown in the presence of the NMDA receptor antagonist, D (-)-amino-7-phosphonovalenic acid (AP5) for 48 hours resulted in no change in mEPSC amplitude or frequency, while 48 hour AMPA receptor blockade via 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) produced the same results observed with TTX (Turrigiano et al., 1998).

Numerous studies since this groundbreaking discovery have focused on determining the mechanism of homeostatic synaptic plasticity, which in contrast to our extensive information on LTP and LTD mechanisms, is still not well understood. Several possibilities exist as to where the site of synaptic strength could be modulated by the nervous system. Presynaptic mechanisms that could



regulate synaptic strength include varying the number of synaptic vesicles being exocytosed or by altering the amount of neurotransmitter being loaded into a single vesicle (Poza and Goda, 2010; Rich and Wenner, 2007). For example, a decrease in network activity could activate a compensatory response mechanism that increases the number of exocytosed vesicles and/or increases the amount of neurotransmitter loaded into each vesicle resulting in an increase of synaptic strength, and vice versa if the network becomes hyperactive (Figure 1, #3 and #1, respectively). The idea of increased vesicle exocytosis after chronic silencing is unlikely to produce a homeostatic change in mEPSC amplitude because frequency of mEPSC events would be expected to increase, which is not usually seen in homeostatic plasticity, and the improbable simultaneous fusion of vesicles would have to occur to produce an increase in mEPSC amplitude. Another potential mechanism for homeostatic plasticity that has received the most consideration and has been extensively studied, involves the bidirectional trafficking of glutamate receptors, specifically AMPA receptors (Figure 1, #2 and #4). Several studies have demonstrated that in response to a variety of chronic activity perturbations, AMPA receptors are trafficked to and from the postsynaptic neuron's membrane in a compensatory manner (Aoto et al., 2008; Correa et al., 2012; Evers et al., 2010; Fu et al., 2011; Gainey et al., 2009; Soden and Chen, 2010; Stellwagen and Malenka, 2006; Wierenga et al., 2005).

### ***GluR1 vs. GluR2 AMPA Receptors in Homeostatic Synaptic Plasticity***

As mentioned earlier, AMPA receptor trafficking to and from the synapse in response to chronic activity perturbations has been well-established to be an important mechanism for the expression of homeostatic plasticity, but discrepancies exist as to which receptor subtypes are involved. Some studies have shown that homeostatic synaptic plasticity requires the increased insertion of the GluR2-containing receptors into the postsynaptic membrane in response to activity deprivation (Cingolani et al., 2008; Gainey et al., 2009) while others have demonstrated an increase in surface GluR1-containing AMPA receptors (Ju et al., 2004; Stellwagen et al., 2005; Sutton et al., 2006; Thiagarajan et al., 2005), and some have shown increases in both GluR1 and GluR2 (Sun and Wolf, 2009; Wierenga et al., 2005). The differences between which subunit is involved may be due to different experimental conditions, such as species (rat vs. mouse), age of the culture (1 week vs. 2 week), type of activity block (TTX vs. receptor block), length of activity block (24 hours vs. 48 hours), or cell type (Table 1), possibly demonstrating the existence of multiple types of homeostatic plasticity. Another possibility is that a two-step process exists where GluR1-containing AMPA receptors are first inserted into the membrane followed by increased expression of GluR2-containing AMPA receptors, as suggested for LTP (Plant et al., 2006).

### ***Glia and Homeostatic Synaptic Plasticity***

More recently, glia have been implicated in homeostatic plasticity (Stellwagen and Malenka, 2006). Glia have long been considered to be merely support cells of neurons in the nervous system, but have now been shown to

release tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) (Beattie et al., 2002) and glypican-4 (Allen et al., 2012) to influence synaptic strength by increasing cell surface expression of GluR1-containing AMPA receptors (Allen et al., 2012; Stellwagen et al., 2005) and glial TNF $\alpha$  has been demonstrated to be essential for homeostatic synaptic plasticity (Stellwagen and Malenka, 2006). Glial release of factors that are capable of increasing synaptic strength in a homeostatic manner by upregulating AMPA receptors into the postsynaptic membrane make them an intriguing candidate as a potential sensor for network activity.

### **Rab3A: A Small GTPase**

Rab proteins are monomeric GTPases that form the largest branch of the Ras superfamily of small GTPases and exist in all eukaryotic cells (Lang and Jahn, 2008; Takai et al., 2001). More than 60 members of Rabs have been identified in mammalian cells (Lang and Jahn, 2008) and these proteins regulate intracellular vesicle trafficking (Takai et al., 2001). Rabs serve as a “switch” by cycling between their active GTP-bound form, where they associate with vesicles and assist in vesicle trafficking to the cell membrane, and their inactive GDP-bound form, where they dissociate from vesicles and localize to the cytosol (Figure 2).

Rab3 is highly expressed in the brain (Pavlos et al., 2010) and Rab3A is overwhelmingly the most abundant isoform located at synapses (Fischer von Mollard et al., 1990). The Rab3 subfamily, which encompasses Rab3A, Rab3B, Rab3C, and Rab3D, has been extensively studied in synaptic function and

secretion, and the four highly homologous isoforms, which share as much as 84% homology (Cheng et al., 2002), have overlapping and redundant functions in vesicle exocytosis (Jahn, 2004; Pavlos and Jahn, 2011). Despite evidence supporting Rab3A's role in synaptic vesicle exocytosis, its exact function is still unclear. Deletion of Rab3A (Rab3A<sup>-/-</sup>) results in mice that are viable and fertile (Schluter et al., 2004), and is not essential for exocytosis but rather may be important for regulating synaptic function and efficiency (Geppert et al., 1994; Geppert et al., 1997). On the other hand, knockout of Rab3A in conjunction with any of the other two isoforms is lethal, while triple Rab3B, Rab3C, and Rab3D knockout animals were viable and fertile (Schluter et al., 2004). Quadruple knockouts develop normally but die shortly after birth due to respiratory failure, have no changes in hippocampal synaptic morphology or mEPSC properties, but do exhibit a 30% decrease in evoked responses of cultured hippocampal neurons that appears to be due to a decrease in release probability (Schluter et al., 2004). Rab3A has also been demonstrated to be necessary for PKA-dependent presynaptic form of LTP at mossy fiber-CA3 synapses in the hippocampus (Castillo et al., 1997) and Rab3A<sup>-/-</sup> hippocampal neurons do not respond to BDNF-induced potentiation of evoked synaptic currents (Thakker-Varia et al., 2001). These results suggest that Rab3A is not critical for vesicle exocytosis, but is required for some forms of plasticity of release probability.

More recently, the Engisch and Rich labs have shown that Rab3A is important for homeostatic synaptic plasticity at the mouse neuromuscular junction (NMJ). The Rab3A *earlybird* mouse (Rab3A<sup>ebd/ebd</sup>), named for its

shortened circadian rhythm, expresses a point mutation, D77G, in the Rab3A guanine nucleotide-binding regions. The mutation decreases the ability of Rab3A to bind both GDP and GTP (Yang et al., 2007) and homeostatic regulation of synaptic strength at the NMJ is abolished in these animals (Wang et al., 2011). Also, the homeostatic increase in synaptic strength at the NMJ is not accompanied by an increase in vesicular acetylcholine transporter, VACHT, or by an increase in postsynaptic acetylcholine receptors (Wang et al., 2011). These results differ from what is generally thought to occur for homeostatic plasticity at central synapses where the increase in synaptic strength is accompanied by an increase in postsynaptic AMPA receptors (Table 1) and/or VGLUT1 expression (Buckby et al., 2006). Interestingly, the ablation of Rab3A (Rab3A<sup>-/-</sup>) at the NMJ did not abolish, but did diminish, the response to activity blockade at these same synapses suggesting that other Rab3A isoforms which are present (Wang et al., 2011) may compensate for the loss of Rab3A. The finding that a presynaptic protein is important for modulating homeostatic synaptic plasticity at the mouse NMJ is surprising since the prevailing view from studies performed in primary cultures of hippocampal and cortical neurons is that homeostatic plasticity is due to the bi-directional regulation and trafficking of postsynaptic AMPA receptors in response to altered network activity. A possible explanation for this could be that the mechanism for homeostatic plasticity is different at the NMJ than in the brain. We will address this possibility in Specific Aims 1 and 2.

### **Rab3A and Disease**

Researchers looking at the synaptic pathology of Alzheimer's Disease (AD) found that Rab3A expression was reduced in both the hippocampus and in the frontal cortex of AD patients and that in these AD patients lower Rab3A levels were found with both increasing duration and severity of dementia (Blennow et al., 1996). Levels of Rab3A in brain tissue from AD patients are reduced in conjunction with the known AD-associated proteins, presenilin-1 and amyloid precursor protein (APP), and the reduction of these proteins are restricted to areas of the brain known to degenerate in AD (Davidsson et al., 2001). More recently, Rab3A has been shown to be required for the assembly of a distinct type of vesicle involved in the transport of APP for cleavage by  $\alpha$ -secretases (Szodorai et al., 2009). Together, these data demonstrate a correlation between Rab3A expression and severity of dementia in AD patients and a functional role of Rab3A activity in regulating the transport of an AD-associated protein, APP.

In addition to AD, Rab3A has been linked to several other disorders of the nervous system. Rab3A expression is reduced in the thalamus, the hippocampus, and the frontal and parietal cortex of post-mortem brains of schizophrenic patients (Davidsson et al., 1999), and is also decreased in the dorsolateral prefrontal cortex of patients with major depressive disorder (Kang et al., 2012). Rab3A has also been demonstrated to regulate the membrane association and dissociation of  $\alpha$ -synuclein, a protein known to accumulate in Lewy bodies in Parkinson's Disease (Chen et al., 2013) suggesting that impairments of  $\alpha$ -synuclein interactions with Rab3A and synaptic vesicles may play a role in neurodegeneration. Deletion of Rab3A rescues the epileptic

phenotype seen in synapsin II knockout mice (Coleman and Bykhovskaia, 2010). The loss of Rab3A impairs glutamatergic excitatory synaptic transmission resulting in the restoration of the excitatory/inhibitory balance that is shifted to an overexcitable phenotype in the absence of synapsin II (Feliciano et al., 2013). Finally, another link between Rab3A and neurological disease is that Rab3A is an mRNA cargo of the translational regulator and RNA transporter, Fragile X mental retardation protein (Miyashiro et al., 2003) and its expression is decreased in cortical neuron synapses of the Fmr1 knockout mouse, a model mouse of Fragile X syndrome (Annangudi et al., 2010).

It is reasonable to believe that if the homeostatic balance and stability in neuronal activity is not maintained, it could lead to or complicate many disorders and diseases of the nervous system, including mental retardation and autism (Ramocki and Zoghbi, 2008; Toro et al., 2010). One example is the Fragile X mental retardation protein, or FMRP, which has been linked to Fragile X syndrome and mental retardation, and has been shown to be essential for retinoic acid-dependent homeostatic increases in synaptic strength in response to neuronal silencing by TTX in the mouse hippocampus (Soden and Chen, 2010).

Disregulation of homeostatic synaptic plasticity has been associated with several diseases and disorders of the nervous system, schizophrenia (Dickman and Davis, 2009), Alzheimer's disease (Pratt et al., 2011), epilepsy (Houweling et al., 2005), and Fragile X mental retardation (Soden and Chen, 2010). Determining the regulatory function of Rab3A, a synaptic protein implicated in

many neurological and psychiatric diseases would have major clinical applications by providing for a new therapeutic target in several nervous system diseases. Here, we present the first evidence that Rab3A, a presynaptic molecule, is required for homeostatic synaptic plasticity. Together these studies, and what is known about Rab3A, suggest Rab3A interacts with multiple neurological disease-associated mechanisms, although whether it is combating or contributing to pathology is unclear. **The fact that Rab3A has been demonstrated to be important for homeostatic synaptic plasticity at the NMJ, leads us to hypothesize that the presynaptic protein, Rab3A, is required for regulating synaptic strength in homeostatic plasticity at central synapses (Specific Aim 1).**

## **II. MATERIALS AND METHODS**

The methods described below are common to all Aims except where noted. Methodology specific to an Aim will be described with that particular Aim.

### **Animals – Rab3A Colony**

Rab3A heterozygous (Rab3A<sup>+/-</sup>) were bred at Wright State University to generate offspring consisting of homozygous wild-type Rab3A (Rab3A<sup>+/+</sup>), homozygous knock-out of Rab3A (Rab3A<sup>-/-</sup>), and Rab3A<sup>+/-</sup>. Animals were



genotyped via Rab3A-targeted PCR of tail DNA using the following primer sequences:

TCC TGT GAC CTC CAA CTG TG (forward)

GGC CCA AAA CTG AGC AAC (reverse)

and the reaction parameters:

1. 96°C for 2:00

2. 94°C for 0:45

3. 58°C for 0:45

4. 72°C for 1:00

(repeat steps 2-4 for a total of 39 cycles)

5. 72°C for 7:00

6. 4°C hold

The PCR products were separated on a 3.5% agarose gel. The above PCR protocol yielded a 178 base-pair Rab3A wild-type amplicon (Figure 3, right lane) and a 170 base-pair Rab3A knock-out amplicon (Figure 3, left lane). Rab3A<sup>+/-</sup> were determined by the presence of both the wild-type and knock-out amplicons (Figure 3, center lane). Rab3A<sup>+/-</sup> progeny were bred to maintain the colony, while final breedings of Rab3<sup>+/+</sup> males with Rab3<sup>+/+</sup> females and Rab3A<sup>-/-</sup> males with Rab3A<sup>-/-</sup> females to produce homozygous litters to be used for experiments. All experiments were performed on neuronal cultures prepared from newborn pups on postnatal day 0 (P0) making it impossible to genotype these animals prior to culture preparation. The homozygous breedings allowed for determination of

genotypes of experimental animals. All animal procedures were approved by Wright State University's Laboratory Animal Care and Use Committee.

### **Animals – Rab3A earlybird Colony**

The breeding strategy for the Rab3A *earlybird* (*ebd*) animals was the same as described above: Rab3A<sup>+/*ebd*</sup> were bred to maintain the colony, while Rab3A<sup>+/<sup>+</sup></sup> pairings and Rab3A<sup>*ebd/ebd*</sup> breedings were mated to produce Rab3A<sup>+/<sup>+</sup></sup> and Rab3A<sup>*ebd/ebd*</sup> progeny, respectively, for experiments. Due to the presence of a single point mutation and the impossibility to simply screen for this via PCR, a more elaborate two-step genotyping protocol was performed utilizing PCR to amplify our region of interest followed by restriction digest of these amplicons with Bsp 1286I to reveal the Rab3A *ebd* point mutation. The PCR protocol used the following primers:

TGA CTC CTT CAC TCC AGC CT (forward)

TGC ACT GCA TTA AAT GAC TCC T (reverse)

in a PCR reaction to amplify the region of DNA that may contain the Rab3A *ebd* point mutation. The reaction parameters were as follows:

1. 94°C for 5:00
  2. 94°C for 0:30
  3. 57°C for 0:40
  4. 72°C for 1:00
- (repeat steps 2-4 for a total of 35 cycles)
5. 72°C for 10:00

## 6. 4°C hold

Two microliters of the 45 µl PCR reaction were ran out on a 1.0% agarose gel and yielded a single product with a size of 557 base-pairs (Figure 4A). The remaining 43 µl of PCR products were saved for further processing in the second step of the genotyping protocol. The presence of a PCR amplicon was used to demonstrate successful DNA amplification for the actual screening process by restriction digest described below. Once it was determined that the PCR was successful, the remaining PCR amplicons were purified and concentrated using the QIAEX II Kit (Qiagen) according to the manufacturers protocol. Briefly, PCR products were transferred to separate tubes and three volumes of Buffer QX1 were added to each tube. Then, 10 µl of QIAEX II were added to each tube and incubated for 10 minutes at room temperature. Samples were vortexed every 2 minutes to maintain the QIAEX II in suspension. After the incubation period, samples were centrifuged at 13,000 rpm for 30 seconds and the supernatant was poured off leaving the pellet. The pellet was then washed twice with 500 µl of Buffer PE and allowed to air dry completely until the pellet was white. After drying, DNA was eluted by adding 20 µl of nuclease-free H<sub>2</sub>O, vortexed to re-suspend the pellet, and incubated at room temperature for 5 minutes. Each sample was then centrifuged at 13,000 rpm for 1 minute and supernatant containing the purified and concentrated DNA was transferred to clean tubes. Samples were then subjected to restriction digest via the restriction enzyme Bsp 1286I (New England Biolabs, Ipswich, MA) per the manufacturers protocol. After restriction digest, samples were ran out on a 3.0% agarose gel yielding 402 and

155 base-pair bands for Rab3A wild-type (*earlybird* colony) (Figure 4B, left lane) and 402, 104, and 51 base-pair bands for the Rab3A *earlybird* mutation (Figure 4B, right lane). Heterozygotes were determined by the presence of all the bands (Figure 4B, center lane).

### **Primary Culture of Mouse Cortical Neurons**

Dissociated cultures of mixed neuronal and glial populations were prepared from P0 Rab3A<sup>-/-</sup> and Rab3A<sup>ebd/ebd</sup> neonates, and compared with cultures from wild-type mice from their respective colonies. Wild-type cultures were produced from each heterozygote colony due to the different genetic backgrounds of the Rab3A KO and Rab3A *ebd* mice. Each culture was prepared from cortices harvested from two animals. Pups were euthanized by decapitation and brains were quickly removed from the skull and pinned at the cerebellum to a chilled Sylgard petri dish. To prevent contamination of cortical neurons with hippocampal neurons, the hippocampi from both hemispheres were removed and discarded and cortices were dissected out and placed into 1 ml of chilled Neurobasal-A media (Gibco), osmolarity adjusted to 270 mOsm, and supplemented with DNase (Fisher ThermoScientific). Tissue digestion was carried out by the addition of papain (Worthington Biochemical) at 20 units/ml and incubated in a 37°C H<sub>2</sub>O bath for 20 minutes with gentle stirring. After digestion, cortices were triturated very gently 3-5 times with a fire-polished Pasteur pipette, filtered through a 100 µm cell strainer (Corning Falcon) to remove chunks of tissue, and transferred to a clean, sterile 15 ml conical tube.

The cell suspension was then centrifuged at 1100 rpm for 2 minutes, supernatant was carefully removed and discarded, and the pellet resuspended with room temperature Neurobasal-A media (270 mOsm), supplemented with 5% fetal bovine serum (FBS; promotes glial growth but is toxic to neurons), 2% B-27 supplement to promote neuronal growth (Gibco), L-glutamine, and gentamicin. Neurons were counted and plated onto 12 mm coverslips pre-coated with poly-L-lysine (Corning) to produce a high density culture. The culture media for the first day (0 DIV) was the same as the above Neurobasal-A media supplemented with FBS, B-27, L-glutamine, and gentamicin, and was switched after 24 hours (1 DIV) to media consisting of Neurobasal-A (270 mOsm), 2% B-27, and L-glutamine and FBS was excluded to avoid its toxic effects on neuronal viability and health (Stellwagen and Malenka, 2006). Cells were maintained in culture for 13-14 DIV and half of the media was changed twice weekly and experiments were performed at 13-14 DIV. Two days prior to experiments, TTX (500 nM; Tocris), a potent Na<sup>+</sup> channel blocker, was added to some cultures to chronically silence all network activity and induce homeostatic synaptic plasticity mechanisms, while sister cultures not receiving TTX treatment served as untreated controls.

### **Whole-Cell Voltage Clamp to Record mEPSCs**

At 13-14 DIV, mEPSCs from TTX-treated and control sister cultures of Rab3A<sup>+/+</sup> or Rab3A<sup>-/-</sup> neurons from the Rab3A<sup>+/+</sup> colony, or Rab3A<sup>+/+</sup> or Rab3A<sup>ebd/ebd</sup> neurons from the Rab3A<sup>+/ebd</sup> colony were recorded from via whole-

cell voltage clamp to assess the role of Rab3A in homeostatic synaptic plasticity. Recordings were taken from pyramidal neurons, which were identified visually as those cells exhibiting a prominent apical dendrite; images were taken of all cells recorded from (Figure 5). Cells were continuously perfused with a solution consisting of (in mM): NaCl (115), KCl (5), CaCl<sub>2</sub> (2.5), MgCl<sub>2</sub> (1.3), dextrose (23), sucrose (26), HEPES (4.2), and pH = 7.2 (Stellwagen and Malenka, 2006). On the day of the experiment, the osmolarity of the media from the cultures was measured (normally 285 – 295 mOsm) and the perfusate osmolarity was adjusted to match the culture osmolarity to guard against osmotic shock to the neurons. To isolate glutamatergic mEPSCs, TTX (500 nM) and picrotoxin (50 μM) were included in the perfusion solution to block action potentials and GABAergic currents, respectively. Patch electrodes (3.5 – 5 MΩ) were filled with an internal solution containing (in mM): K-gluconate (128), NaCl (10), EGTA (1), CaCl<sub>2</sub> (0.132), MgCl<sub>2</sub> (2), HEPES (10), and pH = 7.2. Osmolarity was adjusted to 10 mOsm less than the perfusion solution osmolarity. Neurons were clamped at a voltage of -60 mV using an Axopatch 200B patch-clamp (Axon Instruments) and recorded from for 2 – 5 minutes. Data were collected with Clampex 10.0 (Axon Instruments).

### **Data Analysis of mEPSCs**

Mini Analysis software (Synaptosoft) was used to identify mEPSCs and all events above a threshold of 3 pA were included in the data. We obtained a mean mEPSC amplitude for each neuron and, unless otherwise noted, *n* is the

number of neurons . All data were pooled based on the experimental condition from 2-10 separate cultures. Overall means for each condition were determined from the pooled data and are presented as  $\pm$ SEM for the number of neurons indicated. mEPSC data were compared using an unpaired Student's *t*-test and statistical significance established at  $p < 0.05$ .

For cumulative probability distribution plots of mEPSC amplitude, the first 50 mEPSCs were selected per cell per experimental condition, pooled together, and sorted in ascending order. Experimental mEPSC amplitude distributions were plotted on the same graph as control mEPSC amplitude distributions for comparisons of shifts in distributions.

### **mEPSC Recordings are AMPAergic**

To determine that all events in our recordings of mEPSCs from neurons were AMPAergic in nature and to eliminate the possibility of contamination of our data with NMDA-mediated mEPSCs, we performed whole-cell voltage clamp on wild-type control neurons in the presence of the AMPA receptor blocker, CNQX (10  $\mu$ M). The experiment was done as follows; dissociated cultures of mixed glial and neuronal populations from Rab3A<sup>+/+</sup> mouse cortex were set up as described above, were not chronically treated with TTX, and were recorded from at 13 DIV. Baseline neuronal activity was determined by whole-cell voltage clamp, as described above, for 2 minutes and were performed in the presence of TTX (500 nM, to block action potentials) and picrotoxin (50  $\mu$ M, to block GABA<sub>A</sub> –mediated currents) to isolate mEPSCs. After the 2 minute baseline recordings, the AMPA

receptor blocker, CNQX (10  $\mu$ M), was perfused into the recording chamber for 2 minutes to allow for its action. Once the CNQX was washed in for 5 minutes, mEPSCs were recorded for 5 minutes, followed by a 10 minute wash-out of CNQX, and then recorded from again to get a post-CNQX recording. This was done on 2 separate cultures for a total of 5 cells.

Here we show that all mEPSCs we recorded are AMPAergic. When mEPSCs were recorded in the presence of CNQX, all activity was silenced (Figure 6, middle), and activity was re-established upon removal of the AMPAR block (Figure 6, bottom). The same result was observed in 5 out of 5 cells.

### **III. SPECIFIC AIMS**

Three Specific Aims were tested to elucidate the role of Rab3A in regulating homeostatic synaptic plasticity:

1. Complete loss (Rab3A<sup>-/-</sup>) and mutation of Rab3A (Rab3A<sup>ebd/ebd</sup>) eliminates the homeostatic increase in synaptic strength in dissociated mouse cortical neuron cultures.
2. GluR2-containing AMPA receptors, not GluR1, are increased after chronic TTX blockade, and ablation of Rab3A prevents this increase.
3. Neuronal Rab3A, not glial Rab3A, is responsible for regulating the homeostatic increase in mEPSC amplitude.



**Specific Aim 1: Complete loss (*Rab3A*<sup>-/-</sup>) or mutation of *Rab3A***

***(Rab3A<sup>ebd/ebd</sup>) eliminates the homeostatic increase in synaptic strength in dissociated mouse cortical neuron cultures.***

**Rationale**

As mentioned earlier, the presynaptic GTPase, Rab3A, has been implicated in several forms of synaptic plasticity in the nervous system (Castillo et al., 1997; Thakker-Varia et al., 2001; Wang et al., 2011). Our lab has recently demonstrated that normally functioning Rab3A is necessary for homeostatic synaptic plasticity at the mouse neuromuscular junction (NMJ) and that complete loss of Rab3A results in a blunted homeostatic response to chronic activity silencing via TTX (Wang et al., 2011). Additionally, the homeostatic increase in synaptic strength observed at the mouse NMJ is not accompanied by an increase surface expression of postsynaptic receptors (Wang et al., 2011) as has been observed in many studies of homeostatic plasticity at central synapses (Table 1). Taken together, the lack of change in postsynaptic receptor expression, and that the requirement for a presynaptic protein, Rab3A, in homeostatically-induced increases in synaptic strength at the mouse NMJ, suggest that there may be a presynaptic mechanism involved in the regulation of the activity-dependent increase in miniature endplate currents (mEPCs). One study has provided evidence for presynaptic modulation of miniature inhibitory postsynaptic currents (mIPSCs) in a homeostatic manner. In conjunction with everything discussed thus far involving excitatory synaptic homeostasis,

chronically blocking synaptic activity also triggers a decrease in the amount of inhibition by reducing mIPSC amplitude. This has been shown to be accompanied by a reduction in the amount of postsynaptic GABA<sub>A</sub> receptors in neocortical synapses (Kilman et al., 2002), and by also decreasing presynaptic vesicle filling of GABA via the reduction of the GABA biosynthetic enzyme glutamic acid decarboxylase (GAD65) and the vesicular GABA transporter (VGAT) in hippocampal synapses (Hartman et al., 2006). No presynaptic regulatory role or molecule has been established for the homeostatic regulation of excitatory synapses. Since our lab has previously shown that the Rab3A<sup>ebd/ebd</sup> mutant impairs homeostatic synaptic plasticity at the mouse *NMJ*, we hypothesized that normally functioning Rab3A is required for the homeostatic increase in synaptic strength at *central synapses*.

### Protocol

To test our hypothesis, we used whole-cell voltage clamp to record mEPSCs from cortical pyramidal neurons in control and TTX-treated sister cultures. Culture, electrophysiology, and data analysis were performed as described earlier in the Materials and Methods section. TTX effects on cortical pyramidal neuron mEPSC characteristics were examined on cultures prepared from Rab3A<sup>ebd/ebd</sup> mice and compared to cultures prepared from wild-type mice in that colony; the same comparison was performed on cultures prepared from Rab3A<sup>-/-</sup> cortical pyramidal neurons and cultures prepared from wild-type mice in the same colony. Rab3A<sup>ebd/ebd</sup> and Rab3A<sup>-/-</sup> mice were maintained through

distinct, non-overlapping breeding colonies. Statistical significance was determined as  $p < 0.05$ .

## Results

To test the effect of chronic inactivity and the role of the Rab3A *ebd* mutation on the physiological properties of excitatory synapses, we measured the amplitude and frequency of mEPSCs from TTX-treated (48 hours) and untreated sister cultures of Rab3A<sup>*ebd/ebd*</sup> dissociated cortical pyramidal neurons and compared these to wild-type (Rab3A<sup>*+/+*</sup>) cultures. Untreated Rab3A<sup>*+/+*</sup> neurons had an average amplitude of  $11.0 \pm 0.6$  pA and occurred at a frequency of  $1.15 \pm 0.19$  Hz ( $n = 20$  cells). The amplitude of mEPSCs was significantly increased with chronic silencing of synaptic activity by TTX in Rab3A<sup>*+/+*</sup> neurons ( $15.1 \pm 1.2$ ,  $n = 23$  cells,  $p = .007$  compared to control; Figure 7A left panel, C left, and D), and the TTX effect was blocked in Rab3A<sup>*ebd/ebd*</sup> cultures (untreated,  $14.9 \pm 1.0$  pA vs. TTX-treated  $14.6 \pm 1.1$  pA,  $p = 0.86$ . Control  $n = 22$  cells, TTX  $n = 22$ ; Figure 7A right panel, C right, and D). We did observe that the control mEPSC amplitude of Rab3A<sup>*ebd/ebd*</sup> cultures was increased (14.9 pA) when compared to the control amplitude of Rab3A<sup>*+/+*</sup> cultures (11.0 pA) and that this increase resembled the amplitude of TTX-treated Rab3A<sup>*+/+*</sup> cultures (15.1 pA) (Figure 7D). We cannot rule out that the *ebd* mutation itself produces a TTX-like effect on mEPSC amplitude that obscures the actual TTX-induction of increasing amplitude by achieving maximal synaptic efficacy without TTX, and a similar increase was observed at the NMJ (Wang et al., 2011). However, this may also

be due to culture-to-culture variations in mEPSC amplitude because other labs have shown high variability of mEPSC amplitudes across cultures (Cingolani and Goda, 2008; Gainey et al., 2009; Stellwagen and Malenka, 2006), and we have also seen this high variability in mEPSC amplitudes in our experiments.

Although it is possible that there exist a threshold where basal amplitude is too large that it is no longer able to produce a homeostatic increase, we have data and results from our pooled Rab3A<sup>+/+</sup> cultures (Rab3A KO colony) that show TTX treatment is able to increase mEPSC amplitude to 18.2 pA (Figure 8D); this allows us to believe that the lack of effect of TTX to reflect true inhibition of the homeostatic plasticity mechanism.

The mEPSC frequency of neurons from Rab3A<sup>+/+</sup> mice also increased significantly with chronic TTX treatment (untreated,  $1.15 \pm 0.19$  Hz vs. TTX-treated,  $2.54 \pm 0.55$  Hz. Untreated  $n = 20$ , TTX-treated  $n = 23$ ,  $p = 0.030$ ; Figure 7A left and B), which was blunted in Rab3A<sup>ebd/ebd</sup> neurons (untreated,  $1.64 \pm 0.39$  Hz vs. TTX-treated,  $3.05 \pm 0.80$  Hz,  $p = 0.12$ . Untreated  $n = 22$  neurons, TTX  $n = 22$  neurons; Figure 7A right panel and B). These results demonstrate that normally functioning Rab3A is essential for the homeostatic increase in mEPSC amplitude and frequency.

We next wanted to determine the effect of complete loss of Rab3A (Rab3A<sup>-/-</sup>) on the homeostatic changes on mEPSC physiological characteristics. To test this, we performed the same experiments as done on Rab3A<sup>ebd/ebd</sup> neurons, but using cortical cultures from Rab3A<sup>-/-</sup> mice and their wild-type counterparts. Untreated neurons from Rab3A<sup>+/+</sup> exhibited a significant increase

in both mEPSC amplitude and frequency when compared to sister cultures that were chronically silenced for 2 days by TTX ( $14.0 \pm 0.7$  pA vs.  $18.2 \pm 0.8$  pA,  $P = 0.0004$ , and  $2.03 \pm$  Hz vs.  $4.14 \pm 0.67$  Hz,  $P = .005$ , respectively. Untreated  $n = 26$ , TTX  $n = 20$ ; Figure 8). When the same experiments were performed on Rab3A<sup>-/-</sup> neurons, the TTX-induced increase in mEPSC amplitude was blocked ( $13.5 \pm 0.8$  pA vs.  $14.3 \pm 0.7$  pA,  $P = 0.5$ , control  $n = 22$ , TTX  $n = 21$ ; Figure 7A, C, and D). Ablation of Rab3A also blunted the homeostatic increase in mEPSC frequency ( $2.94 \pm 0.54$ Hz vs  $3.70 \pm 1.13$  Hz,  $P = 0.5$ , control  $n = 22$ , TTX  $n = 21$ ; Figure 7A and B). These results strongly suggest that Rab3A is necessary for the physiological expression of increased synaptic strength as a result of chronic TTX induced homeostatic synaptic plasticity in mouse cortical neurons.

## Discussion

Chronic perturbations in the synaptic activity of neuronal networks are known to cause global compensatory changes in synaptic strength between neurons. Our findings here demonstrate the essential role of a presynaptic protein, Rab3A, in regulating the homeostatic increase in synaptic strength at AMPAergic synapses. Our data demonstrate that either a mutation to obstruct Rab3A's GTPase activity or complete loss of the protein inhibits the homeostatic increase in mEPSC amplitude and blunts the response on mEPSC frequency in dissociated mouse cortical pyramidal neurons.

It is well-established that chronic silencing of neuronal networks leads to an increase in mEPSC amplitude and several molecules have been identified to

support the mechanism of homeostatic postsynaptic modulation of glutamatergic AMPA receptors, including glial release of the pro-inflammatory cytokine, TNF $\alpha$ , which increases expression of surface AMPARs (Beattie et al., 2002), up-regulation of all-trans retinoic acid (RA) synthesis to increase surface GluR1-containing AMPA receptors (Aoto et al., 2008) and the requirement of Fragile X mental retardation protein (FMRP) in the RA-dependent upregulation of GluR1 AMPARs (Soden and Chen, 2010), loss of Arc, an immediate-early gene associated with information encoding in the brain, results in increased basal GluR1 surface expression and mEPSC amplitude and prevents the TTX-induced increase in amplitude (Shepherd et al., 2006), and  $\beta$ 3-integrin, a cell adhesion molecule involved in the endocytosis of AMPARs, is necessary for the homeostatic increase in cell membrane GluR2-containing AMPA receptor numbers (Cingolani et al., 2008).

As mentioned previously, glial release of factors in response to chronic activity deprivation have also been shown to be important for increasing synaptic strength by increasing the amount of postsynaptic GluR1-containing AMPA receptors. To this date, no known presynaptic mechanism or molecules have been shown to be involved in the homeostatic increase in synaptic strength, but our discovery that Rab3A is necessary for this process provides a novel physiological role for Rab3A at central synapses and could provide the first evidence of a presynaptic mechanism in homeostatic synaptic plasticity.

There are several potential Rab3A-dependent mechanisms for regulating the increase in synaptic strength in neurons (Figure 9). Rab3A could work

presynaptically by increasing the amount of glutamate loaded into synaptic vesicles in response to chronic activity silencing, as has been seen with GABA in inhibitory neurotransmission (Hartman et al., 2006). The increase in the amount of transmitter being released per vesicle would subsequently activate more postsynaptic AMPA receptors causing an increase in mEPSC amplitude. Another potential Rab3A-dependent mechanism is that Rab3A homeostatically controls the release of a presynaptic signaling molecule that travels across the synaptic cleft to activate the increased insertion of postsynaptic AMPARs, resulting in increased synaptic strength. A third potential mechanism for Rab3A in homeostatic synaptic plasticity located in the postsynaptic cell could be that Rab3A, known to be involved in the trafficking of vesicles, acts from its location within the postsynaptic neuron by shuttling AMPAR-loaded vesicles to the synapse when neuronal activity is deprived, allowing for increased synaptic strength. The last mechanism whereby Rab3A could regulate the homeostatic increase in synaptic strength is that Rab3A in glia, in response to persistent activity suppression, modulates the release of a glial-derived factor, such as TNF $\alpha$ , that signals to increase postsynaptic expression of AMPARs.

Due to the fact that malfunctioning of neuronal activity homeostasis has been implicated in neurological and psychiatric diseases, determining Rab3A's role in regulating the enhancement of synaptic efficacy in this process would provide a better understanding of this mechanism and molecular signals. This better understanding could provide for new drug targets and therapies. As a first step, we address whether loss of Rab3A prevents increased neurotransmitter

loading in synaptic vesicles or increased postsynaptic AMPA receptors in Specific Aim 2.

**Specific Aim 2: *Rab3A<sup>-/-</sup> blocks the homeostatic increase in postsynaptic expression of surface GluR2-containing AMPA receptors in response to chronic activity blockade.***

Specific Aim 2 was designed to determine the role of Rab3A in the mechanism of homeostatic synaptic plasticity. As mentioned previously, there are several possibilities for the site of Rab3A's mechanism of action in response to chronic activity deprivation, such as: 1. acting presynaptically to alter synaptic vesicle neurotransmitter concentration, 2. regulating the presynaptic release of a molecule that signals to the postsynaptic neuron to increase AMPAR numbers, 3. working in the postsynaptic neuron to control the increased trafficking of AMPA receptors to the synapse, or 4. modulating the release of a glial-derived factor that subsequently signals to increase AMPAR numbers in the postsynaptic neuron. The scope of this Aim was to determine if Rab3A is involved in a presynaptic mechanism by assessing the amount of transmitter loaded into synaptic vesicles by using a glutamate competitive antagonist or if it is required to induce the necessary postsynaptic manipulations to AMPAR numbers, and more specifically, which AMPAR subunit, GluR1 or GluR2, it may be regulating. We have partitioned Specific Aim 2 into 3 subsections to answer these questions: A. Presynaptic Mechanism: Neurotransmitter Concentration, B. Postsynaptic



Mechanism: GluR1-containing AMPARs, and C. Postsynaptic Mechanism: GluR2-containing AMPARs.

### **A. Presynaptic Mechanism: Neurotransmitter Concentration**

*Is Rab3A mediating a presynaptic homeostatic mechanism? Links between presynaptic homeostasis, BDNF, and Rab3A.*

Our results demonstrating that the synaptic vesicle protein, Rab3A, is necessary for the expression of increased synaptic strength in homeostatic synaptic plasticity could lead to the first line of evidence of a presynaptic mechanism and the identification of a presynaptic molecule linked to the process of homeostatically increasing excitatory synaptic strength. As mentioned earlier, homeostatic modulation of neurotransmitter concentration has been shown in inhibitory synaptic transmission in hippocampal neurons. Chronic activity blockade via TTX triggers a decrease in mIPSC amplitude due to TTX-induced downregulation of the GABA synthesizing enzymes, glutamic acid decarboxylase 67 (GAD67) (Lau and Murthy, 2012) and GAD65 (Hartman et al., 2006), and decreased vesicle loading of GABA has been demonstrated by increased sensitivity to a competitive GABA antagonist (Hartman et al., 2006). This TTX-driven homeostatic decrease in inhibitory synaptic transmission is also regulated by brain-derived neurotrophic factor (BDNF) via TrkB receptor signaling as demonstrated by the prevention of the activity deprivation effects on GABAergic synapses when cultures were co-treated with BDNF (Swanwick et al., 2006).

BDNF application to hippocampal neurons has been shown to increase Rab3A protein expression and to potentiate synaptic activity; the BDNF-induced potentiation of synaptic activity is abolished in neurons lacking Rab3A (Thakker-Varia et al., 2001). These data illustrate BDNF's role in modulating synaptic activity in decreasing inhibitory synaptic transmission by reducing GABA concentration in synaptic vesicles resulting in increased excitatory synaptic transmission and increased synaptic strength. The fact that BDNF signaling is able to 1.) vary the amount of the inhibitory transmitter, GABA, loaded into vesicles in an activity-dependent manner, 2.) that Rab3A is required for a BDNF-driven potentiation of excitatory synaptic activity, and 3.) that no evidence to date has implicated a homeostatic mechanism at excitatory synapses of modulation of glutamate concentration in vesicles makes it reasonable to believe that BDNF may be signaling through Rab3A to regulate the amount of glutamate loaded into vesicles at excitatory synapses.

*Is Rab3A mediating a presynaptic homeostatic mechanism? The role of Rab3A in fusion pore kinetics.*

Another possibility for Rab3A to modulate the amount of neurotransmitter present in the synaptic cleft is by regulating synaptic vesicle exocytotic fusion pore kinetics. Fusion pores are channels that form at the initial phase of exocytosis sometimes resulting in full-collapse vesicle fusion and release of all transmitter content of that vesicle, or, failure to achieve full fusion and releasing varying amounts of transmitter into the synaptic cleft (Ales et al., 1999). In

adrenal chromaffin cells, loss of Rab3A results in abnormally slow-rising and long duration pre-spike feet of amperometric events, corresponding to fusion of single catecholamine-containing vesicles (Wang et al., 2008). As pre-spike feet are a measure of fusion pore dynamics (Wightman et al., 1995), this result suggests Rab3A may regulate the amount of transmitter available in the cleft in a fusion pore-dependent manner.

*Can a change in glutamate release result in a different mEPSC amplitude?*

Variations in glutamate concentration in the synaptic cleft have been shown to be the primary source of variability in evoked EPSC amplitude. It has also been concluded that vesicular packets of transmitter are not able to saturate postsynaptic receptors (Liu et al., 1999), leaving room for fluctuations in glutamate concentration as a source of variability in mEPSC amplitudes (Wu et al., 2007), leaving the possibility for these fluctuations in vesicular glutamate content to contribute to a presynaptic mechanism in homeostatic plasticity.

Since Rab3A localizes to synaptic boutons and associates with vesicles, is necessary for BDNF-induced synaptic plasticity, exhibits the ability to regulate fusion pore kinetics, and we have now shown its requirement for the expression of homeostatic plasticity, we hypothesized that Rab3A is required for a homeostatic increase in the amount of neurotransmitter released into the synaptic cleft.

Protocol

To test our hypothesis that Rab3A is homeostatically regulating the amount of transmitter released into the synaptic cleft, we used the compound,  $\gamma$ -D-glutamylglycine ( $\gamma$ -DGG), to investigate this possibility.  $\gamma$ -DGG is a glutamate receptor antagonist that has a low affinity for AMPA receptors (Watkins et al., 1990) and is a rapidly dissociating competitive antagonist of glutamate (Liu et al., 1999), allowing for the investigation of cleft glutamate concentrations. If variations in mEPSC amplitude are due to variations in the amount of glutamate in the cleft, there should be an inverse correlation in the amount of  $\gamma$ -DGG inhibition and the size of mEPSC amplitude. In other words, if large amplitude mEPSCs are due to increased glutamate concentration, then these larger amplitude mEPSCs would show decreased sensitivity to  $\gamma$ -DGG because the increased glutamate in the synaptic cleft would out-compete  $\gamma$ -DGG for AMPA receptor binding, and vice versa.

We used whole-cell voltage clamp to record mEPSCs from untreated and TTX-treated (48 hours) sister cultures of Rab3A<sup>+/+</sup> (Rab3A colony) neurons before and after  $\gamma$ -DGG application. Cultures were prepared as described in the Materials and Methods section. Electrophysiology was performed as described earlier, except basal recordings were done for 4 minutes, followed by a 2 minute wash-in with  $\gamma$ -DGG (500  $\mu$ M) added to perfusate, and then a 4 minute recording from the same neuron in the presence of  $\gamma$ -DGG. This was done on untreated and TTX-treated sister cultures of Rab3A<sup>+/+</sup> neurons and results compared between the 2 experimental conditions.

Neuronal mEPSC data were analyzed as described in the Materials and Methods section. Mean percent reduction of mEPSC amplitudes for each cell in untreated and TTX-treated conditions in response to  $\gamma$ -DGG were calculated, then pooled together for each condition to obtain an overall mean percent reduction for untreated and TTX-treated conditions, and were compared. Statistical significance was established as  $p < 0.05$ . Also, each individual neuron's percent reduction was plotted against its basal average mEPSC amplitude to determine whether any relationships exist between initial amplitude or TTX treatment and the amount of reduction on mEPSC amplitude observed with  $\gamma$ -DGG inhibition.

## Results

When we compared the average amount of reduction on mEPSC amplitudes of control and TTX-treated neurons triggered by competitive antagonism via  $\gamma$ -DGG, we found there was no difference between the two conditions ( $26.1 \pm 3.4\%$  reduction in control vs.  $26.6 \pm 4.8\%$  in TTX-treated,  $p = 0.9$ . Control  $n = 12$ , TTX  $n = 9$ ; Figure 10A.). This could be due to the lack of a strong TTX effect on mEPSC amplitude as a result of control neuron's exhibiting a large basal mean amplitude prior to  $\gamma$ -DGG application ( $16.2 \pm 1.0$  pA vs.  $18.4 \pm 1.4$  pA,  $p = 0.21$ , control  $n = 12$ , TTX  $n = 9$ , data not shown). This could obscure any differences we may have seen in the amount of  $\gamma$ -DGG inhibition because the initial amplitudes of control and TTX-treated neurons were very similar. To address this, we then examined each cell individually to determine if

large amplitude neurons were less sensitive to  $\gamma$ -DGG inhibition and if small amplitude cells were more sensitive to  $\gamma$ -DGG, and whether this was dependent on TTX-treatment. We discovered that the percent reduction on mEPSC amplitude was independent of initial amplitude size or of a TTX effect (Figure 10B) as we observed individual neurons which had small mEPSC amplitudes and were relatively insensitive to  $\gamma$ -DGG (Figure 10B and C, top, note, the smallest amplitudes were all from untreated cultures), an untreated neuron with a large mEPSC amplitude was highly sensitive to  $\gamma$ -DGG (Figure 10B and C, middle), and a TTX-treated neuron with a large mEPSC amplitude was highly sensitive (Figure 10B and C, bottom). These surprising results that larger amplitudes are more sensitive to  $\gamma$ -DGG is opposite of what would be expected if large mEPSC amplitude was a result of increased neurotransmitter. This strongly suggests that the amount of  $\gamma$ -DGG inhibition is irrespective of mEPSC amplitude or chronic TTX treatment.

### Discussion

Our surprising results that  $\gamma$ -DGG was less effective in inhibiting small amplitudes and had varying effects on mEPSC inhibition regardless of mEPSC size or TTX effect (Figure 10B and C) strongly suggests that sensitivity to  $\gamma$ -DGG is independent of amplitude and chronic network silencing. This is the opposite result expected if variations in mEPSC amplitude were due to transmitter concentration. If transmitter concentration was equivalent in small and large amplitude cells, we would expect to see the same amount of reduction of

mEPSC amplitudes of untreated and TTX-treated neurons. Instead, our results indicate that, 1.) very large amplitude must be due to increased receptors and not transmitter concentration, and, 2.) when increased receptors are present they may serve as a sink for glutamate, lowering its concentration in the synaptic cleft and making the neuron more sensitive to  $\gamma$ -DGG.

These results are strong evidence that presynaptic modulation of neurotransmitter is not the mechanism for homeostatic increases in mEPSC amplitude in the face of chronic network silencing. They also strongly suggest that neurotransmitter concentration within synaptic vesicles is widely varying, potentially contributing to the high variability seen in mEPSC amplitudes. Also, neurons with large mean amplitudes actually appear to be contacted by presynaptic neurons that have, on average, vesicles with less neurotransmitter content because some of the largest amplitude cells exhibited the most sensitivity to competitive antagonism by  $\gamma$ -DGG (Figure 10B). This heightened sensitivity to  $\gamma$ -DGG in large amplitude neurons may also provide evidence for an increase in postsynaptic AMPA receptors, which has been well-established to contribute to activity-dependent increases in mEPSC amplitude. Our results presented here argue against an involvement of a presynaptic mechanism modulating an increase in neurotransmitter concentration to enhance synaptic efficacy in response to chronic activity deprivation, and may point towards the postsynaptic mechanism of modulating AMPA receptors and Rab3A's involvement in regulating their activity-dependent increase.

## **B. Postsynaptic Mechanism: GluR1-containing AMPA Receptors**

### Rationale

Bidirectional AMPA receptor trafficking via vesicles to and from the synapse has been well documented and occurs in an experience-dependent manner to influence synaptic strength (Heynen et al., 2000; Takahashi et al., 2003), and during homeostatic synaptic plasticity (Wierenga et al., 2005). Knockout of the GluR1 subunit impairs the regulation of synaptic strength and distance-dependent synaptic scaling in the CA1 region of the mouse hippocampus (Andrasfalvy et al., 2003). Since AMPA receptors are known to be shuttled to and from the cell surface and Rab3A has long been known as a small GTPase that cycles between association and dissociation with GTP and synaptic vesicles (Fischer von Mollard et al., 1990) to traffic vesicular cargo for exocytosis (Matteoli et al., 1991), it is reasonable to believe that Rab3A could also be involved in the postsynaptic transport of AMPA receptor-loaded vesicles.

As mentioned previously, modifications to postsynaptic AMPA receptor levels are now known to play an essential role for the expression of homeostatic synaptic plasticity, but there is disagreement between the involvement of the GluR1 subunit or the GluR2 subunit. Several studies have demonstrated the increased insertion of GluR1-containing AMPA receptors in response to chronic activity blockade (Table 1). Another study showed that BDNF contributes to the bidirectional cell surface expression of both GluR1-containing AMPA receptors and GluR2-containing AMPA receptors in nucleus accumbens medium spiny



neurons (Reimers et al., 2014), and 2 other studies, 1 on nucleus accumbens neurons (Sun and Wolf, 2009) and the other on visual cortical cultures (Wierenga et al., 2005), also showed that prolonged activity silencing increases cell surface expression of both GluR1 and GluR2. Preferential homeostatic upregulation of GluR2-containing AMPA receptors has also been shown (Cingolani et al., 2008) and the GluR2 subunit has been demonstrated to be required for synaptic scaling, a type of homeostatic plasticity (Gainey et al., 2009). Despite the discrepancy in which AMPA receptor subunit is responsible for the expression of homeostatic plasticity, the majority of evidence supports the upregulation of the GluR1 subunit after prolonged activity deprivation (Table 1).

Based on our results here demonstrating Rab3A's requirement for the expression of increased mEPCS amplitude in homeostatic synaptic plasticity (Figure 7 and 8) and that increased transmitter concentration does not account for this increase in amplitude (Figure 10), we hypothesized that Rab3A is regulating the increase in synaptic efficacy in response to chronic activity silencing by modulating the increase in postsynaptic AMPA receptors, specifically the GluR1 subtype since overwhelming evidence has shown the homeostatic increase in GluR1 expression. Although we know of no postsynaptic involvement of Rab3A in the trafficking vesicles to the membrane, it is reasonable to believe that Rab3A could be involved in this process due to its already known role in trafficking synaptic vesicles for exocytosis and in the secretory pathway. The fact that BDNF-induced plasticity is reliant on Rab3A and BDNF has recently been shown to adjust surface AMPA receptor levels in a

homeostatic manner, allows for the possibility that Rab3A is affecting postsynaptic AMPA receptor expression in an activity-dependent manner to increase synaptic strength, or provides a link between Rab3A and receptor trafficking. A potential role for Rab3A to modulate receptor trafficking could be by regulating the release of a neuronal factor, such as BDNF, which in turn regulates receptors postsynaptically. To our knowledge, this would provide a novel role for Rab3A and the first evidence of its postsynaptic function.

### Protocol

Here, we tested whether GluR1 is increased homeostatically using 2 approaches: 1.) immunohistochemistry of surface GluR1 expression, and 2.) a selective GluR1 antagonist, NASPM (a synthetic analog of Joro spider toxin). We examined GluR1 receptor levels in untreated and TTX-treated (2 days) sister cultures, and some of these cultures we also measured mEPSC amplitudes to establish a physiological correlate with the staining for GluR1 expression. NASPM experiments were performed on untreated and TTX-treated (2 days) sister cultures and all recordings were done before and after NASPM.

### Immunohistochemistry.

Primary cultures of wild-type mouse cortical neurons and TTX treatment were prepared as described in the Materials and Methods section. Surface expression of GluR1 was examined on 2 week old cultures of untreated and TTX-treated sister cultures of wild-type pyramidal neurons. Antibody to GluR1

(see Table 2 for information regarding antibodies) was added to unfixed coverslips in the culture media in which each coverslip was grown (so not to introduce any changes in osmolarity or factors released by glia and neurons that coverslips have been exposed to while in culture), 5% donkey serum. Coverslips were incubated for 45 minutes in a 37°C, 5% CO<sub>2</sub> incubator. Neurons were then rinsed with PBS, fixed for 10 minutes in 4% paraformaldehyde at room temperature, rinsed with PBS, and then rinsed with donkey serum, and incubated 1 hour at room temperature with Cy5 (649) donkey anti-rabbit secondary antibody (Jackson ImmunoResearch) in 5% donkey serum. The cells were then permeabilized with 0.2% saponin before incubating in MAP2 and VGLUT1 antibodies, to label cell bodies and dendrites, and presynaptic glutamatergic terminals, respectively. Cells were then incubated in secondary antibodies for 45 minutes, rinsed, and then mounted to slides using VectaShield mounting medium (Vector Labs) to reduce photobleaching. Slides were stored at 4°C and imaged within a few days by confocal microscopy.

Confocal images were acquired on an Olympus FV1000 confocal microscope using FluoView 2.1 software. The objective was 60X (oil immersion), with a numerical aperture of 1.35. A single image of a visually identified pyramidal neuron was acquired before applying a 5X zoom to acquire a stack of images of the primary dendrite (section thickness = 0.5 microns). Where possible, ten cells and their primary dendrites were imaged per coverslip per treatment condition (untreated and TTX-treated). Imaging parameters of PMT and laser percent were adjusted for each laser to maximize brightness without

causing saturation. The same parameters were used for untreated and TTX-treated coverslips from a single experiment.

### Image Analysis

The confocal z-stack of the dendrite was flattened using Image Pro 6 (Cybernetics), and an area of interest (AOI) defined that included the dendrite plus a fixed amount outside the dendrite (to capture receptor clusters on spines) that was defined by using the process command morphological filter to “swell” the dendrite. To quantify the size, intensity, and integrated intensity of receptor clusters, a threshold was set (400-600 depending on overall brightness; the same threshold was used for untreated and TTX-treated in a single experiment) and the count command applied to automatically circle receptor clusters that were located within the AOI. To exclude multiple clusters that had been circled as a single cluster due to their proximity, clusters larger than 0.8 microns (check by looking at several dendrites and finding out what is the biggest size) were not included in the analysis. Cluster area, intensity, and integrated intensity were acquired, and the average across the clusters determined for each dendrite, and then an overall average across dendrites were calculated. The effect of TTX was assessed in 2 ways. For the total number of 7 experiments, the untreated and TTX-treated means for each experiment were compared with a student’s t-Test, where  $n$  = number of experiments. For 2 experiments in which physiology was performed on the same day as immunohistochemistry, data are presented as individual experiments.

### Synaptic Analysis

In ImagePro 6, dendrite (color 1), VGLUT1-positive presynaptic terminal (color 2) and receptor antibody-positive clusters (color 3) are visible simultaneously in the composite image, facilitating identification of synaptic pairs that are located on or close to the dendrite. To find a synaptic pair, the slice number is cycled up and down the confocal stack, and coexisting pre- and postsynaptic sites circled by hand to create the AOIs. The stack number for each AOI is noted for use later. When all of the synaptic pairs for an individual dendrite have been circled, the AOI list is saved. Next, the single color image for receptors is viewed, each AOI is placed on the image, on the correct confocal slice, and the count tool is used to automatically circle the receptor cluster. The measurement values (area, intensity, and sum) are copied to an Origin file, with all of the data for a single dendrite accumulated in one worksheet, for calculating an average for the dendrite.

### Physiological Blockade of GluR1-mediated mEPSCs by NASPM

N-naphthyl acetylspermine (NASPM) is a selective antagonist of GluR2-lacking AMPA receptors (Koike et al., 1997). It has been shown to abolish homeostatic induced increases in mEPSC amplitudes induced in chick motoneurons *in vivo* by activity blockade via the sodium channel blocker, lidocaine, for 2 days (Garcia-Bereguian et al., 2013), which suggests that in that preparation GluR2-lacking AMPA receptors are entirely responsible for the

activity-deprivation induced increase in mEPSC amplitude. Cultures were prepared and whole-cell voltage clamp was performed as described in the Materials and Methods section. Baseline recordings were performed for 2 minutes and the same patched cell was then perfused with a high  $K^+$  solution containing NASPM (same solution used for baseline recordings except  $[KCl] = 25$  mM,  $[NaCl] = 95$  mM, and NASPM = 20  $\mu$ M) for 45 seconds. NASPM is an activity-dependent drug, so perfusion with the high  $K^+$  solution caused the cell to depolarize, inducing a high frequency of evoked synaptic events (personal observation) which allows glutamate binding to open GluR2-lacking AMPA receptors. After 45 seconds, perfusion was returned to our normal extracellular solution used for the basal recordings, but with NASPM (20  $\mu$ M) and the cell was allowed to recover its membrane potential to resting levels. After 5 minutes, a 5 minute recording of mEPSCs was then acquired in the presence of NASPM. For each cell, there was a baseline recording and a NASPM recording.

Mean pre-NASPM recordings for each cell in each condition (untreated vs. TTX-treated) were pooled together to acquire an overall mean amplitude and frequency for untreated and TTX-treated conditions. These were then compared to determine a TTX effect on mEPSC amplitude and frequency. NASPM's effect on mEPSC amplitude and frequency was determined by comparing the different conditions, untreated vs. TTX-treated, before and after NASPM. TTX effects on mEPSC characteristics was established by an unpaired Student's *t*-test comparing overall means of untreated and TTX-treated pre-NASPM recordings, and NASPM's effect on each neuron's mEPSC amplitude and frequency was

compared before and after NASPM using a paired sample  $t$ -test. Statistical significance was determined as  $p < 0.05$ . In addition, we calculated the mean amplitude for the largest 25 events from each neuron recorded from and plotted the percent change in amplitude after NASPM against the pre-NASPM amplitude for each individual neuron.

## Results

### *Effect of activity blockade on GluR1 receptor levels: immunohistochemistry*

We stained untreated and TTX-treated Rab3A<sup>+/+</sup> cultures for GluR1 to determine if the homeostatic increase in mEPSC amplitude seen with chronic activity silencing is accompanied by an increase in cell surface expression of GluR1-containing AMPA receptors. Example images of GluR1 expression in untreated and TTX-treated cultures are shown in Figure 11 (these images are only intended to demonstrate immunohistochemistry of MAP2, VGLUT1, and GluR1 expression; not to show any differences between untreated and TTX-treated conditions). We found that overall total size of GluR1 puncta did not change between control and TTX-treated sister cultures of Rab3A<sup>+/+</sup> pyramidal neurons ( $0.32 \pm 0.06 \mu\text{m}^2$  vs.  $0.31 \pm 0.05 \mu\text{m}^2$ , paired  $t$ -Test  $p = 0.9$ ,  $n = 7$  cultures; Figure 12A, left). There was also no difference GluR1 cluster intensity in untreated and TTX-treated Rab3A<sup>+/+</sup> cultures (untreated,  $988 \pm 51$  arbitrary units (a.u.) vs.  $991 \pm 56$  a.u., paired  $t$ -Test  $p = 0.8$ ,  $n = 7$  cultures; Figure 12B, left). When we analyzed individual experiments, we found a variable effect of

chronic TTX silencing on GluR1 size in Rab3A<sup>+/+</sup>; out of 7 cultures, 2 showed a dramatic increase in GluR1 puncta size (60% and 78%; Figure 12A, right), 1 showed a decrease (-53%, Figure 12A, right), and 4 showed very modest changes (12%, -10%, -14%, and -16%; Figure 12A, right). Analysis of individual experiments on surface GluR1 intensity showed there were essentially little to no change in intensity (Figure 12B, right). These results left us with the questions of whether there are multiple mechanisms involved in the expression of homeostatic synaptic plasticity as has been suggested by the disagreement over GluR1 or GluR2 involvement (see Table 1, addressed in Specific Aim 2C), or, sometimes there is not a TTX effect on mEPSC amplitude (addressed below).

Because we have seen some individual wild-type cultures that have not exhibited a measurable physiological increase in mEPSC amplitude after TTX blockade, and the differences in GluR1 immunohistochemistry from experiment to experiment varied widely (Figure 12A, right), we examined GluR1 immunohistochemistry in 2 cultures for which we also performed electrophysiological measurements on the same day. Recordings were taken from neurons in untreated and TTX-treated cultures and coverslips from the same cultures were subjected to immunohistochemistry for surface GluR1 expression; this would allow us to determine a correlation between a physiological TTX-induced increase in mEPSC amplitude and cell surface GluR1 expression. To our knowledge, recordings of mEPSCs from the same cultures that are used investigate AMPA receptor expression by immunohistochemistry have never been done before. For imaging and GluR1 immunohistochemistry



analysis, we presented all dendrites imaged from each of the 2 experiments along with the mean across dendrites for each experiment. We also added an analysis of synaptically identified GluR1 clusters, selected by their proximity to presynaptic VGLUT1.

In the first physiology-GluR1 immunohistochemistry correlation experiment (Experiment 1), we observed an increase in mEPSC amplitude after chronic TTX treatment, that did not reach significance (untreated,  $13.8 \pm 2.2$  pA vs. TTX-treated,  $17.7 \pm 1.8$  pA,  $p = 0.19$ . Untreated  $n = 7$ , TTX treated  $n = 8$  neurons; Figure 13A). Because the TTX effect is of small magnitude, the number of cells needed to observe a statistically significant effect is usually  $>8$ , which is why we perform multiple days of recordings. Despite this caveat, Experiment 1 showed a strong trend towards a TTX-induced increase in mEPSC amplitude that we felt would be accompanied by at least a trend toward increased surface GluR1 expression after chronic TTX-treatment if GluR1 were the AMPA receptor subunit responsible for the homeostatic increase in mEPSC amplitude. The results were clear; there was no change in total GluR1 puncta size after chronic silencing (untreated,  $0.28 \pm 0.02 \mu\text{m}^2$  vs. TTX-treated,  $0.27 \pm 0.02 \mu\text{m}^2$ ,  $p = 0.87$ . Untreated  $n = 10$ , TTX-treated  $n = 9$ ; Figure 13B, left) or in total GluR1 intensity (untreated,  $741 \pm 19$  a.u. vs.  $738 \pm 5$  a.u.,  $p = 0.86$ . Untreated  $n = 10$ , TTX-treated  $n = 9$ ; Figure 13B, right), and the same result was seen with synaptic GluR1 cluster size (untreated,  $0.15 \pm 0.02 \mu\text{m}^2$  vs. TTX-treated,  $0.17 \pm 0.02 \mu\text{m}^2$ ,  $p = 0.44$ . Untreated  $n = 10$ , TTX-treated  $n = 10$ ; Figure 13C, left) and synaptic

GluR1 intensity (untreated,  $657 \pm 22 \mu\text{m}^2$  vs. TTX-treated,  $663 \pm 17 \mu\text{m}^2$ ,  $p = 0.83$ . Untreated  $n = 10$ , TTX-treated  $n = 10$ ; Figure 13C, right).

The second time we performed the physiology-GluR1 immunohistochemistry experiment (Experiment 2), we had a strong TTX-induced increase in mEPSC amplitude (untreated,  $13.5 \pm 1.5 \text{ pA}$  vs. TTX-treated,  $19.1 \pm 1.8 \text{ pA}$ ,  $p = 0.034$ . Untreated  $n = 7$ , TTX-treated  $n = 6$ ; Figure 14A). Because of the strong TTX effect on mEPSC amplitude in Experiment 2, we would expect a very clear increase in GluR1 expression if GluR1 upregulation were mediating the homeostatic increase mEPSC amplitude. Again, the results were clear; surface GluR1 expression in Experiment 2 did not increase (puncta size: untreated,  $0.27 \pm 0.04 \mu\text{m}^2$  vs. TTX-treated,  $0.22 \pm 0.01 \mu\text{m}^2$ ,  $p = 0.18$ ; Figure 14B, left. Intensity: untreated,  $761 \pm 17 \text{ a.u.}$  vs. TTX-treated,  $750 \pm 8 \mu\text{m}^2$ ,  $p = 0.58$ ; Figure 14B, right. Untreated  $n = 10$ , TTX-treated  $n = 10$ ). There were also no homeostatic changes in synaptic GluR1 expression (puncta size: untreated,  $0.13 \pm 0.01 \mu\text{m}^2$  vs.  $0.13 \pm 0.02 \mu\text{m}^2$ ,  $p = 0.83$ ; Figure 14C, left. Intensity:  $615 \pm 13 \text{ a.u.}$  vs.  $631 \pm 17 \text{ a.u.}$ ,  $p = 0.45$ ; Figure 14C, right. Untreated  $n = 10$ , TTX-treated  $n = 10$ ).

In summary, when physiology-GluR1 expression correlation experiments were performed, we found no evidence of an increase in GluR1 levels, despite there being a clear demonstration of a TTX-induced increase in mEPSC amplitude in the same experiment. These results suggest that either GluR1 is not involved in the homeostatic response to chronic activity deprivation, which is

unlikely due to the prevalence of studies showing GluR1 increased homeostatically, or there multiple homeostatic mechanisms exist.

We also performed immunohistochemistry experiments for GluR1 expression in response to chronic TTX treatment in Rab3A<sup>-/-</sup> cultures. Interestingly, there was a modest homeostatic increase in GluR1 levels despite the loss of Rab3A (untreated,  $0.30 \pm 0.03 \mu\text{m}^2$  vs. TTX-treated,  $0.33 \pm 0.03 \mu\text{m}^2$ ,  $p = 0.40$ ,  $n = 7$  cultures; Figure 15A), and analysis of individual cultures revealed that 2 out of 4 experiments demonstrated the modest homeostatic increase in GluR1 puncta size (Figure 15B). These data from Rab3A<sup>-/-</sup>, like the immunohistochemistry experiments examining homeostatic changes in GluR1 levels in wild-type cultures, again show that a subset of cultures show a modest homeostatic increase in GluR1 expression, and that loss of Rab3A does *not* prevent this increase.

#### *Effect of activity blockade on GluR1 levels: selective antagonism by NASPM*

Because immunohistochemistry experiments revealed no changes in GluR1 levels after chronic TTX-treatment even when a homeostatic increase in mEPSC was observed (Figures 13 and 14), it is possible that immunohistochemistry was not sensitive enough, that our methods of analysis failed to pick up a genuine change, or multiple mechanisms can increase mEPSC amplitude in a homeostatic manner. Since several other studies have shown increased GluR1 levels after chronic activity silencing (Table 1), we used another independent measure that should reveal if there were a significant

increase in GluR1 homomers, a selective antagonist of GluR2-lacking receptors that has previously been used to demonstrate a selective contribution of GluR2-lacking receptors to a homeostatic increase in synaptic strength in chick spinal cord neurons (Garcia-Bereguiain et al., 2013). If the increase in synaptic strength after chronic activity-deprivation is due to increased expression of GluR1 homomeric receptors, we would expect NASPM application to abolish the increase in mEPSC amplitudes in TTX-treated neurons. The most striking effect of NASPM we found was on mEPSC frequency. We were surprised to find that the homeostatic increase in mEPSC frequency (untreated pre-NASPM,  $1.88 \pm 0.41$  Hz vs. TTX-treated pre-NASPM,  $4.23 \pm 0.90$  Hz, untreated  $n = 16$  neurons, TTX  $n = 13$  neurons,  $p = 0.017$ ; Figure 16C) was abolished after NASPM (untreated pre-NASPM,  $1.88 \pm 0.41$  Hz vs. TTX-treated NASPM,  $2.40 \pm 0.61$ , untreated  $n = 16$  neurons, TTX  $n = 13$  neurons,  $p = 0.47$ ; Figure 16C). Paired analysis before and after NASPM for both untreated and TTX-treated conditions showed significant decreases in mEPSC frequency in response to NASPM inhibition, although the effect was more pronounced in TTX-treated cultures (untreated: pre-NASPM,  $1.88 \pm 0.41$  Hz vs. NASPM,  $1.46 \pm 0.36$  Hz,  $n = 16$ ,  $p = 0.027$ ; Figure 16C, black bars. TTX-treated: pre-NASPM,  $4.23 \pm 0.90$  Hz vs. NASPM,  $2.40 \pm 0.61$  Hz,  $n = 13$ ,  $p = 0.006$ ; Figure 16C, green bars, and E).

Our result showing that NASPM inhibition of GluR1 AMPA receptors blocks the homeostatic increase in frequency is highlighted in an individual neuron from a TTX-treated culture in Figure 16. Figure 16D shows a portion of a recording before and after NASPM where the frequency was decreased by

43.4%, but there was no effect of NASPM on decreasing that same cell's mEPSC amplitude (pre-NASPM, 18.6 pA vs. NASPM, 18.9 pA; Figure 16B, right - the histogram plot in Figure 16B labeled "Neuron 2" is from the same cell as the example trace in Figure 16D). If GluR1-containing AMPA receptors were solely responsible for the homeostatic increase in amplitude, we would have expected this cell, which exhibited a TTX-induced increase in mean amplitude (18.6 pA), to have a mean amplitude after NASPM (18.9 pA) that resembled the mean amplitude of untreated cultures (~ 13 pA). Our findings that inhibition of GluR1-containing AMPA receptors via NASPM has varying effects on blocking the TTX-induced increase in mEPSC amplitude but does block the homeostatic increase in frequency, suggests that postsynaptic patches of AMPA receptors are not heterogeneous mixtures of different receptor subunits, but rather are exclusively regions of either GluR1-containing or GluR2-containing receptors. To our knowledge, this is a novel finding.

Whole-cell voltage clamp of pyramidal neurons from Rab3A<sup>+/+</sup> cultures showed an increase in mEPSC amplitude in response to chronic TTX-treatment when pre-NASPM recordings from untreated and TTX-treated (48 hours) were compared (untreated,  $13.1 \pm 0.9$  pA vs. TTX,  $16.6 \pm 1.0$  pA,  $p = 0.016$ , 26.7% increase in amplitude. Untreated  $n = 16$  cells, TTX-treated  $n = 13$  cells; Figure 16A). However, NASPM treatment did not affect the TTX-induced increase in amplitude (untreated to TTX-treated % increase after NASPM = 26.6%), and they did not resemble untreated, pre-NASPM recordings as would be expected if the homeostatic increase in mEPSC amplitude were due to increased GluR1

expression (untreated pre-NASPM,  $13.1 \pm 0.9$  pA vs. TTX NASPM,  $15.7 \pm 0.9$  pA,  $p = 0.06$ . Untreated pre-NASPM  $n = 16$ , TTX-treated NASPM  $n = 13$ ; Figure 16A, CON pre bar vs. TTX NASPM bar).

These results are unlikely to be due to a lack of effect of NASPM because paired analysis of individual neurons showed very slight, but not significant, reductions in mEPSC amplitude after NASPM (untreated pre-NASPM  $13.1 \pm 0.9$  pA vs. untreated NASPM  $12.4 \pm 0.7$  pA,  $p = 0.29$ ,  $n = 16$ ; Figure 16A, black bars. TTX-treated pre-NASPM  $16.6 \pm 1.0$  pA vs. TTX-treated NASPM  $15.7 \pm 0.9$  pA,  $n = 13$ ,  $p = 0.18$ ; Figure 16A, green bars). In addition, we observed a clear and consistent effect of NASPM on frequency, regardless of its effect on amplitude (Figure 16C)

Further examination of the amplitude histograms in cells in which NASPM caused a reduction in amplitude, suggests that NASPM may be mainly acting on the largest amplitude events (Figure 16B, Neuron 1, compare pre and post NASPM). To examine whether NASPM had a differential effect on the largest amplitude events, we determined the mean of the top 25 events, before and after NASPM, and calculated a percent change. While there was not a significant difference in magnitude of NASPM effect in untreated and TTX-treated cells, when we plotted the percent change as a function of the pre-NASPM mean of the largest 25 events in each cell, there was a weak ( $R^2 = 0.19$ ) but significant ( $p = 0.01$ ) relationship, with the cells that had the largest “top 25” means having the largest decreases with NASPM. Since the cells with the largest “top 25” means are TTX-treated cells, this result suggests there is some contribution, albeit

small, of GluR2-lacking receptors to the TTX-induced increase in mEPSC amplitude in our cultures.

### Discussion

Our evidence of neurons with larger events trending to towards increased NASPM sensitivity, coupled with the result that 2 out of 7 of the GluR1 immunohistochemistry experiments on wild-type cultures and 2 out of 4 experiments on Rab3A<sup>-/-</sup> cultures showed a homeostatic increase in GluR1 expression, points toward some involvement of GluR1 in homeostatic plasticity that is not Rab3A-dependent in our cultures, but clearly cannot fully explain the homeostatic increase in mEPSC amplitude. Based on these data using 2 independent approaches, immunohistochemistry and pharmacology, we demonstrate that the TTX-induced increase in mEPSC amplitude in our preparation is not due to a selective increase GluR1 receptor expression. It was very surprising to us that NASPM did block the homeostatic increase in frequency, a novel finding suggesting homogeneity of receptor type expression in individual receptor patches.

### **C. Postsynaptic Mechanism: GluR2-containing AMPA Receptors**

#### Rationale

Since our thorough approach using 2 independent methods of immunohistochemistry and pharmacology did not expose a strong role for GluR1

in the homeostatic increase in mEPSC amplitude (Specific Aim 2B), we wanted to test whether an increase in GluR2-containing AMPA receptor number was responsible for the increase in synaptic strength in our cultures, and if Rab3A was involved in this process. Although evidence predominantly has implicated GluR1 upregulation as the source for the homeostatic increase in mEPSC amplitude, other labs have shown that GluR2 is responsible for the increase in mEPSC amplitude after chronic network silencing (Table 1).

### Protocol

Cultures, physiology, and immunohistochemistry experiments were performed as described in Specific Aim 2B, with the exception that GluR2 immunohistochemistry analysis looked at synaptic GluR2 expression only due to the extreme amount of GluR2 expression that made it impossible to ensure that individual clusters could be automatically circled across the whole dendrite in a flattened image. In some immunohistochemistry experiments had electrophysiology performed on the same day. Physiology statistics were performed as described in the Materials and Methods section and immunohistochemistry statistics were performed as outlined in Specific Aim 2.

### Results

We performed immunohistochemistry on untreated and TTX-treated sister cultures of Rab3A<sup>+/+</sup> and Rab3A<sup>-/-</sup> cortical pyramidal neurons to test if GluR2 expression is upregulated with chronic activity deprivation and whether loss of



Rab3A affects GluR2 expression patterns. Example images of GluR2 immunohistochemistry are in Figure 17 (these are simply for demonstration of synaptic sites, and not intended to show any TTX-induced changes in GluR2 expression). Chronic TTX treatment produced a modest increase in synaptic GluR2 cluster size in pyramidal neurons from Rab3A<sup>+/+</sup> cultures (untreated,  $0.83 \pm 0.08 \mu\text{m}^2$  vs. TTX-treated,  $0.97 \pm 0.10 \mu\text{m}^2$ ,  $p = 0.18$  (paired t-Test). Untreated  $n = 5$ , TTX-treated  $n = 5$ ; Figure 18A, left), and this modest effect on GluR2 expression was blocked in Rab3A<sup>-/-</sup> cultures (untreated,  $0.75 \pm 0.17 \mu\text{m}^2$  vs. TTX-treated,  $0.74 \pm 0.07 \mu\text{m}^2$ ,  $p = 0.94$  (paired t-Test). Untreated  $n = 4$ , TTX-treated  $n = 4$ ; Figure 18C, left). As we observed with GluR1 expression after chronic TTX treatment (Figure 12), homeostatic changes in GluR2 expression also had experiment to experiment variation (Figure 18A, right, and 18C, right), although to a lesser degree than GluR1. Interestingly, 4 out of 5 experiments on Rab3A<sup>+/+</sup> cultures showed a homeostatic increase in synaptic GluR2 puncta size while the other experiment showed a decrease in GluR2 puncta size (Figure 18A, right), and 3 out of 4 experiments on Rab3A<sup>-/-</sup> cultures exhibited a decrease after TTX while the other experiment showed a dramatic increase GluR2 expression (Figure 18C, right). GluR2 intensity did not change in response to TTX treatment either in Rab3A<sup>+/+</sup> cultures (untreated,  $731 \pm 38$  a.u. vs. TTX-treated,  $735 \pm 27$  a.u.,  $p = 0.77$  (paired t-Test). Untreated  $n = 5$ , TTX-treated  $n = 5$ ; Figure 18B) or in Rab3A<sup>-/-</sup> cultures (untreated,  $715 \pm 38$  a.u. vs. TTX-treated,  $735 \pm 14$  a.u.,  $p = 0.64$  (paired t-Test). Untreated  $n = 4$ , TTX-treated  $n = 4$ ; Figure 18D).

Although these results suggest Rab3A may have some involvement in the homeostatic upregulation of GluR2-containing AMPA receptors, the 2 experiments that had the opposite expected effects on changes in GluR2 expression after TTX was puzzling. In some of these experiments, we also performed electrophysiology, so we wanted to determine, as we did with GluR1, if there was a correlation between a homeostatic increase in mEPSC that was accompanied by an increase in GluR2 expression in the same culture. Of the 5 Rab3A<sup>+/+</sup> cultures we performed GluR2 immunohistochemistry on, we also recorded mEPSCs in 3 experiments. In Experiment 1 (Rab3A<sup>+/+</sup>), a homeostatic increase in mEPSC amplitude (28.3%) was observed (not significant,  $p = 0.19$ ; Figure 19A) and was accompanied by a 47.5% increase in synaptic GluR2 puncta size (not significant,  $p = 0.13$ ; Figure 19B, left). Experiment 2 (Rab3A<sup>+/+</sup>) also showed a correlation between a TTX-induced increase in mEPSC amplitude (12.0%, not significant,  $p = 0.58$ ; Figure 19C) and increased synaptic GluR2 expression after chronic silencing (20.6%, not significant,  $p = 0.25$ ; Figure 19D, left). (Note: the modest size of the homeostatic effect is such that we do not expect individual experiments with these  $n$ 's to reach significance). The fact that the modest increase in amplitude observed in Experiment 2 (Rab3A<sup>+/+</sup>) was accompanied by a similarly modest increase in GluR2 expression, and that the large homeostatic increase in mEPSC amplitude seen in Experiment 1 (Rab3A<sup>+/+</sup>) was accompanied by a large increase in GluR2 expression in suggests that in these 2 cultures GluR2 upregulation is responsible for the homeostatic increase in mEPSC amplitude.

The third physiology-GluR2 immunohistochemistry experiment on Rab3A<sup>+/+</sup> cultures (Rab3A<sup>+/+</sup>, Experiment 3) produced a surprising result where a 17.2% homeostatic increase in amplitude (not significant,  $p = 0.09$ ; Figure 19E) was accompanied by a 9.2% decrease in synaptic GluR2 puncta size (not significant,  $p = 0.58$ ; Figure 19F, left). Another study has also shown that a homeostatic decrease in GluR2-containing AMPA receptors after chronic activity deprivation results in increased mEPSC conductance which is mediated by a resulting increase in the proportion of GluR1 homomers (Garcia-Bereguain et al., 2013), which are reported to have a larger conductance than GluR1-GluR2 (Swanson et al., 1997). Such a shift from heteromers to GluR1 homomers could possibly explain why a modest homeostatic increase in mEPSC amplitude might be accompanied by an decrease in GluR2.

Physiology-GluR2 immunohistochemistry correlation experiments were also performed on cortical cultures from Rab3A<sup>-/-</sup> mice to test whether loss of Rab3A affects the homeostatic expression of GluR2 AMPA receptors. In Experiment 1 (Rab3A<sup>-/-</sup>), we saw a slight increase in mEPSC amplitude with chronic TTX treatment (11.4%, not significant,  $p = 0.53$ ; Figure 19G) that was accompanied by a very large increase in GluR2 puncta size (146.8%,  $p = 0.0002$ ; Figure 19H, left). It is puzzling that this very large homeostatic increase in GluR2 expression (146.8%) was not accompanied by a correlative very large increase in mEPSC amplitude (11.4%), but large homeostatic increases in GluR2 expression (>140%) have been reported by other labs (Sun and Wolf, 2009; Wierenga et al., 2005). Also, it must be noted that in Experiment 1 (Rab3<sup>-/-</sup>) the untreated GluR2

puncta size is much smaller (Figure 19H, left) than any of the other GluR2 immunohistochemistry experiments examining puncta size, leaving us with the question of whether there truly was a very large increase in synaptic GluR2 puncta size or if this increase was exaggerated due to the unexplained low GluR2 expression in the untreated condition.

Experiments 2 and 3 from Rab3A<sup>-/-</sup> cultures produced similar results to each other where the loss of Rab3A not only blocked the homeostatic increase in mEPSC amplitude, but also caused it to decrease slightly (Experiment 2: -6.5%, Figure 19I, left; Experiment 3: -11.2%, Figure 19K, left), and the decreases in amplitude were accompanied by correlative decreases in synaptic GluR2 puncta size (Experiment 2: -7.2%, Figure 19J, left; Experiment 3: -11.3%, Figure 19L, left). The results from Experiments 2 and 3 on Rab3A<sup>-/-</sup> cultures suggest that in these two cultures that the loss of Rab3A blocked the homeostatic increase in GluR2 expression resulting in the inhibition of the homeostatic increase in mEPSC amplitude.

### Discussion

Our results examining the role of GluR2 expression in response to chronic network silencing showed that in some cultures GluR2 levels are homeostatically increased and that the loss of Rab3A appears to block their increase which results in the inhibition of the homeostatic response on mEPSC amplitude. However, in Experiment 3 on Rab3A<sup>+/+</sup> cultures (Figure 19E and F), where a homeostatic increase in mEPSC amplitude was accompanied by a *decrease* in

GluR2 expression, strongly suggests that multiple mechanisms may be involved in the expression of homeostatic synaptic plasticity, as has been suggested by others (Garcia-Bereguian et al., 2013) (see also Table 1).

**Specific Aim 3: Neuronal Rab3A, not glial Rab3A, is responsible for regulating the homeostatic increase in mEPSC amplitude.**

Rationale

We have shown in Specific Aim 1 that Rab3A is essential for the homeostatic increase in synaptic strength (Figures 7 and 8). As mentioned earlier, several possibilities exist as to the location of where Rab3A may be acting to exert its regulatory action to increase mEPSC amplitude in a homeostatic manner (Figure 9).

Mounting evidence suggests that glial-neuronal interactions are complex and important for normal nervous system function (Haydon, 2001). As mentioned earlier, glia have been shown to be important for enhancing excitatory neurotransmission by releasing factors such as glypican-4 and TNF $\alpha$  to increase synaptic strength, and TNF $\alpha$  has been demonstrated to be essential for homeostatic synaptic plasticity. Both of these molecules produce their effect by increasing cell surface expression of GluR1-containing AMPA receptors (Allen et al., 2012; Stellwagen and Malenka, 2006). Since Rab3A is expressed in glia (Madison et al., 1996), it is reasonable to believe that glial Rab3A may mediate the release of activity-dependent factors, such as TNF $\alpha$ , from glia in response to

activity deprivation, that signal to increase AMPA receptor insertion into the postsynaptic membrane. The scope of Specific Aim 3 was to determine whether neuronal or glial Rab3A was modulating the homeostatic increase in synaptic strength.

### Protocol

To test our hypothesis that glial Rab3A is necessary for the increase in synaptic strength in homeostatic synaptic plasticity, we cultured Rab3A<sup>+/+</sup> neurons onto Rab3A<sup>-/-</sup> or Rab3A<sup>+/+</sup> glial feeder layers, and Rab3A<sup>-/-</sup> neurons onto Rab3A<sup>+/+</sup> glia (Figure 20A).

### Preparation of Glial Feeder Layers

Glial feeder layers were prepared from the cortices of P0-P2 Rab3A<sup>+/+</sup> or Rab3A<sup>-/-</sup> mouse pups. Cortices were dissected and cells were dissociated as described previously in the Materials and Methods section. Cell suspensions of mixed neuronal and glial populations were plated onto glass coverslips pre-coated with poly-L-lysine (Corning) in Dulbecco's Modified Eagle Media (Life Technologies, Invitrogen) supplemented with 5% FBS (to promote glial proliferation and to kill neurons), L-glutamine, and gentamicin, and maintained in an incubator at 37°C, 5% CO<sub>2</sub>; cultures were maintained in this manner for up to 1 month until seeded with neurons. Culture media was replaced after 24 hours, and subsequent media changes were made twice weekly replacing half of the culture media with fresh media. Feeder layers were not used for neuronal

seeding until all native neurons were gone and glial cells approached 100% confluency (visually inspected).

### Plating of Neurons on Glial Feeder Layers

Cortical neurons were obtained as described in the Materials and Methods section. Upon acquisition of a pellet containing mixed neuronal and glial populations, the pellet was resuspended in Neurobasal-A (270 mOsm) containing B27 (2%, to promote neuronal growth), L-glutamine, and 5-fluorodeoxyuridine (FdU, a mitotic inhibitor). Addition of FdU was used to prevent glial growth and contamination of the glial feeder layer with new glia, promoting only neuronal growth on the feeder layers (this media was used for the maintenance of these cultures and all subsequent media changes were with this media). Glial culture media was removed from the feeder layer cultures, and the cell suspension was plated onto the glial feeder cultures. Culture strategy is outlined in Figure 15A. At 1 DIV all of the culture media was removed and replaced with fresh Neurobasal-A media described above, and half of the media was replaced twice per week for all subsequent media changes. Cultures were maintained in a 37°C, 5% CO<sub>2</sub> incubator for 13-14 days.

### Electrophysiology

TTX treatment of cultures, whole-cell voltage clamp, data acquisition, data analysis, and statistics of mEPSCs from cortical pyramidal neurons were performed as described in the Materials and Methods section.

## Results

To test whether glial Rab3A was responsible for the homeostatic increase in synaptic strength, we cultured wild-type neurons onto Rab3A<sup>-/-</sup> glial feeder layers and recorded mEPSCs from untreated cultures and TTX-treated (48 hours) sister cultures and compared mEPSC amplitude and frequency between the two conditions. We first performed these experiments on cultures of wild-type neurons on wild-type glial feeder layers to demonstrate that this new culturing strategy of glial feeder layers exhibited a homeostatic increase in mEPSC amplitude (untreated,  $13.3 \pm 0.5$  pA vs. TTX-treated,  $16.7 \pm 1.2$  pA,  $p = 0.022$ . Untreated  $n = 17$ , TTX-treated  $n = 20$ ; Figure 20B, left and middle). Despite not being significant, these cultures trended towards a homeostatic increase in frequency (untreated,  $2.54 \pm 0.57$  Hz vs. TTX-treated,  $3.48 \pm 0.64$  Hz,  $p = 0.29$ . Figure 20B, right) – (mEPSC data from Specific Aim 1 showing initial results of a homeostatic increase in synaptic strength in our original culturing strategy of wild-type neurons are provided in Figure 20C for reference). Rab3A<sup>+/+</sup> neurons plated on Rab3A<sup>-/-</sup> glia still showed a robust homeostatic response on mEPSC amplitude (untreated,  $13.3 \pm 1.0$  pA vs. TTX-treated,  $18.8 \pm 1.4$  pA,  $p = 0.005$ . Untreated  $n = 11$ , TTX-treated  $n = 11$ ; Figure 20D, left and middle), and trended towards a homeostatic increase in frequency (untreated,  $2.01 \pm 0.41$  Hz vs. TTX-treated,  $4.47 \pm 1.53$  Hz,  $p = 0.14$ ; Figure 20D, right). This result indicates that Rab3A is *not* required in the glia. We also performed the same experiment on cultures of Rab3A<sup>-/-</sup> neurons on wild-type glia, and the



homeostatic response was reduced and no longer significant (untreated,  $15.2 \pm 1.1$  pA vs. TTX-treated,  $16.9 \pm 0.7$  pA,  $p = 0.23$ . Untreated  $n = 14$ , TTX-treated  $n = 11$ ; Figure 20E, left and middle), but there was a slight decrease in mEPSC frequency that was not significant (untreated,  $4.47 \pm 1.21$  Hz vs.  $3.02 \pm 0.70$  Hz,  $p = 0.35$ ; Figure 20E, right) – (mEPSC data from Specific Aim 1 showing initial results of loss of Rab3A blocking the homeostatic increase in synaptic strength in our original culturing strategy are provided in Figure 20F for reference). These results further confirm that Rab3A localized within neurons is playing a key role in regulating homeostatic synaptic plasticity.

## Discussion

These results, that loss of *glial* Rab3A does *not* block the homeostatic increase in mEPSC amplitude, and loss of neuronal Rab3A *does* block the TTX-induced increase in amplitude, focuses the potential possibilities for Rab3A-dependent homeostatic increases in mEPSC amplitude to two possible mechanisms. The first possibility is that Rab3A is working in the presynaptic neuron, not to modulate transmitter content within synaptic vesicles as we originally postulated (see Specific Aim 2A, and Figure 10), but rather by regulating the anterograde signaling of the presynaptic release of neurotrophic factors that travel across the synaptic cleft that subsequently signal to increase AMPA receptor levels in the postsynaptic membrane (Figure 9, #2). The second possibility is that Rab3A is located in the postsynaptic neuron and is involved in increasing the trafficking of AMPA receptors for insertion into the postsynaptic

membrane in response to chronic activity deprivation (Figure 9, #3). Both of these possibilities would provide new insight and better understanding of the homeostatic mechanism for increasing mEPSC amplitude. To our knowledge, there is currently no evidence for anterograde signaling from the presynaptic to the postsynaptic neuron to homeostatically increase AMPA receptor numbers, and despite Rab3A's known role in vesicle trafficking, the predominant belief is that its action is in the presynaptic neuron and not the postsynaptic neuron. If Rab3A were located within the postsynaptic neuron and regulated AMPA receptor insertion, it would provide a novel physiological role for Rab3A in the postsynaptic neuron as well as establishing a new molecule involved in the homeostatic upregulation of AMPA receptor levels.

#### **IV. DISSERTATION SUMMARY**

##### **Specific Aim Conclusions**

##### **Specific Aim 1**

1. Loss of Rab3A blocks the homeostatic increase in synaptic strength.
2. Mutation of Rab3A blocks the homeostatic increase in synaptic strength.

##### **Specific Aim 2**

##### **A. Presynaptic Mechanism – Transmitter Concentration**

1.  $\gamma$ -DGG sensitivity is independent of mEPSC amplitude and of TTX effect.

2. Neurons with large mean amplitudes appear to have less neurotransmitter or more receptors because the neuron with the largest mean amplitude was the most sensitive to  $\gamma$ -DGG.
3. Neurotransmitter concentration varies widely within vesicles.

#### B. Postsynaptic Mechanism – GluR1-containing AMPA Receptors

1. NASPM inhibition of the homeostatic increase in mEPSC frequency suggests that postsynaptic patches of receptors may not be heterogeneous, but rather exclusively GluR1 or GluR2-containing.
2. GluR1-containing AMPA receptors have some involvement in the homeostatic increase in mEPSC amplitude.
3. The TTX-induced increase in mEPSC amplitude in our preparation is not due to a selective increase GluR1 receptor expression.
4. Loss of Rab3A does *not* prevent a modest homeostatic increase in GluR1 levels.
5. Multiple mechanisms may exist in the manifestation of the homeostatic increase in mEPSC amplitude.

#### C. Postsynaptic Mechanism – GluR2-containing AMPA receptors

1. The homeostatic increase in GluR2 expression appears more consistently than an increase in GluR1, but GluR2 involvement cannot fully explain the homeostatic increase in mEPSC amplitude.
2. Loss of Rab3A may block the homeostatic increase in GluR2 levels.

3. Multiple mechanisms may exist for the manifestation of homeostatic increase in mEPSC amplitude.

### Specific Aim 3

1. Loss of *glial* Rab3A does *not* block the homeostatic increase in mEPSC amplitude.
2. Loss of *neuronal* Rab3A *does* block the homeostatic increase in mEPSC amplitude.

Homeostatic synaptic plasticity provides synaptic circuits with the ability to maintain stable functioning when faced with destabilizing perturbations in synaptic activity that may arise from other plasticity mechanisms, such as LTP and LTD. Since the discovery of homeostatic plasticity in 1998, much effort has been devoted to exposing the mechanism underlying the nervous system's ability to compensate for chronic perturbations in synaptic activity. Consistent in many studies is that the compensatory increase in synaptic strength in response to chronic network silencing is accompanied by an increase in postsynaptic AMPA receptors, but discrepancies exist as to whether GluR1 or GluR2 levels are changed (see Table 1). Based on our findings presented here, we believe that multiple mechanisms exist and no single mechanism can explain the manifestation of the homeostatic change in synaptic strength. For example, in our experiments we cannot conclude a selective regulation of GluR1 or GluR2 because both appear to contribute. Despite our evidence suggesting there is not

a selective homeostatic increase in GluR1 vs. GluR2-containing AMPA receptors, we have strongly demonstrated that the loss or the mutation of Rab3A prevents the homeostatic increase in synaptic strength.

In Specific Aim 1 we definitively demonstrated that Rab3A is essential for the manifestation of the homeostatic increase in mEPSC amplitude and that the location of Rab3A's action in homeostatic plasticity is in the neuron and not in the glia (Specific Aim 3). When we attempted to determine the mechanism of homeostatic plasticity so that we could then determine if loss of Rab3A disrupted that mechanism, we ruled out a presynaptic mechanism (Specific Aim 2A) and focused on postsynaptic modulation of AMPA receptors, specifically GluR1 and GluR2-containing AMPA receptors (Specific Aim 2B and C), as substantial evidence suggests an upregulation of AMPA receptors in response to chronic network activity silencing (Table 1).

Initially we did not see an overall homeostatic increase in GluR1 expression, but when we examined individual experiments for GluR1 involvement, we could not rule out some involvement of GluR1-containing AMPA receptors because we observed a small subset of cultures that did exhibit a homeostatic increase in GluR1 expression (Figure 12A, right), and this modest homeostatic increase in GluR1 levels was not prevented with loss of Rab3A. Electrophysiological experiments with pharmacological blockade of GluR1-containing AMPA receptors via NASPM further confirmed that there is a weak involvement of GluR1 receptors in increasing mEPSC amplitude as neurons with the largest "top 25" mean trended towards increased NASPM-sensitivity (Figure 16D).

Since upregulation of GluR1 receptors after chronic TTX treatment seemed to have only a minor homeostatic role in our cultures, we tested whether GluR2-containing AMPA receptor levels were increased after chronic activity silencing. We discovered that GluR2 expression increased modestly with TTX-treatment (Figure 18A) and that this modest increase in GluR2 expression was blocked in Rab3A<sup>-/-</sup> cultures (Figure 18C). Despite the homeostatic increase in GluR2 expression being slight, we feel it correlates with our physiology data because homeostatic increases in mEPSC amplitude are also small in magnitude. When physiology was coupled with GluR2 immunohistochemistry experiments to determine whether a correlation exists between increased mEPSC amplitude and increased GluR2 expression and if Rab3A is involved, we found that 2 out of 3 Rab3A<sup>+/+</sup> cultures and 1 Rab3A<sup>-/-</sup> culture demonstrated a relationship between a homeostatic increase in amplitude and GluR2 expression (Figure 19A, B, C, and D), and that the other 2 experiments on Rab3A<sup>-/-</sup> cultures blocked both the homeostatic increase in amplitude and GluR2 levels (Figure 19I, J, K, and L). The Rab3A<sup>-/-</sup> culture that exhibited both an increase in amplitude and GluR2 expression could be attributed to falling within the realm of an experimental variability that is large in comparison to homeostatic changes that are small in magnitude. In the Rab3A<sup>+/+</sup> culture that showed a slight homeostatic increase in amplitude despite a slight decrease in GluR2 expression (Figure 19E and F), we believe this may hint at another mechanism of homeostatic plasticity as suggested by another lab where a decrease in GluR2-containing AMPA receptors shifts the ratio in favor of GluR1 receptors resulting in increased

conductance that subsequently increase mEPSC amplitude (Garcia-Bereguian et al., 2013).

With all things considered, our data and the data previously published are consistent with the presence of multiple mechanisms that can accomplish homeostatic synaptic plasticity. This would explain the divergence in the field about GluR1 vs. GluR2 involvement in homeostatic plasticity, and 2 studies have shown that *both* GluR1 and GluR2 are homeostatically upregulated proportionally (Table 1). Another possibility for the variability in finding GluR1 or GluR2 involvement in homeostatic plasticity, is that there could exist 2 phases, as suggested for LTP (Plant et al., 2006), where the early initiation phase of the homeostatic mechanism could involve insertion of GluR1 receptors, which are eventually replaced by incorporation of GluR2 receptors into the membrane for the maintenance of homeostatic plasticity. This could also explain why we did not always see a homeostatic increase in GluR2 expression and saw an increase in GluR1 levels in a small subset of experiments. Protocols for activity blockade in previous studies range from 24 hours to 72 hours with the majority of studies using a 24 hour or 48 hour block of activity (Table 1). The majority of those experiments that used a 24 hour block to induce homeostatic plasticity observed increased GluR1 expression, while most of the studies that showed a homeostatic increase in GluR2 receptors blocked network activity for 48 hours (Table 1). In our experiments, we blocked network activity via TTX treatment of cultures for 2 days, and if there are phasic roles for GluR1 and GluR2 in the initiation and maintenance of homeostatic plasticity, respectively, it may explain

why we saw a more consistent homeostatic increase in GluR2 expression since we performed our experiments during what would be the phase of GluR2 involvement. The 2 experiments where we saw increased GluR1 expression, or the experiment where GluR2 did not increase, could be due to biological variability around when the initiation phase segues into the maintenance phase.

Our results show that both GluR1 and GluR2 can increase in response to chronic activity deprivation, but which predominates appears to vary from culture to culture, and possibly, from cell to cell. In our preparation, a homeostatic increase in GluR2 was more consistent, and with the exception of a strange outlier with an unusually low level of GluR2 expression in the untreated culture (Figure 18G and H), loss of Rab3A prevented the TTX-induced upregulation of GluR2 receptors, but not GluR1 receptors. Based on our results that GluR1 and GluR2 are *both* likely contributing to the manifestation of homeostatic synaptic plasticity, that loss of Rab3A appears to prevent the homeostatic increase in GluR2 but *not* GluR1 receptors, and evidence of a biphasic mechanism in LTP involving GluR1 and GluR2 AMPA receptors (Plant et al., 2006), we believe that homeostatic plasticity occurs through a biphasic mechanism that is Rab3A-dependent. We propose a model (Figure 21) that a chronic decrease in network activity induces (Phase 1, ~ day 1) the homeostatic response by triggering glial release of TNF $\alpha$  that signals to the postsynaptic neuron to increase GluR1-containing AMPA receptors resulting in an increase in synaptic strength. This increase in synaptic strength is maintained (Phase 2, ~ day 2) by initiating the Rab3A-dependent release of BDNF that signals to the postsynaptic neuron to



replace GluR1-containing AMPA receptors with GluR2-containing AMPA receptors. Our model demonstrates a novel biphasic mechanism for the manifestation of homeostatic plasticity, and most importantly, whatever the range of underlying mechanisms of altered synaptic strength that can be triggered by chronic activity blockade, synaptic strength cannot be modulated in the absence of Rab3A.

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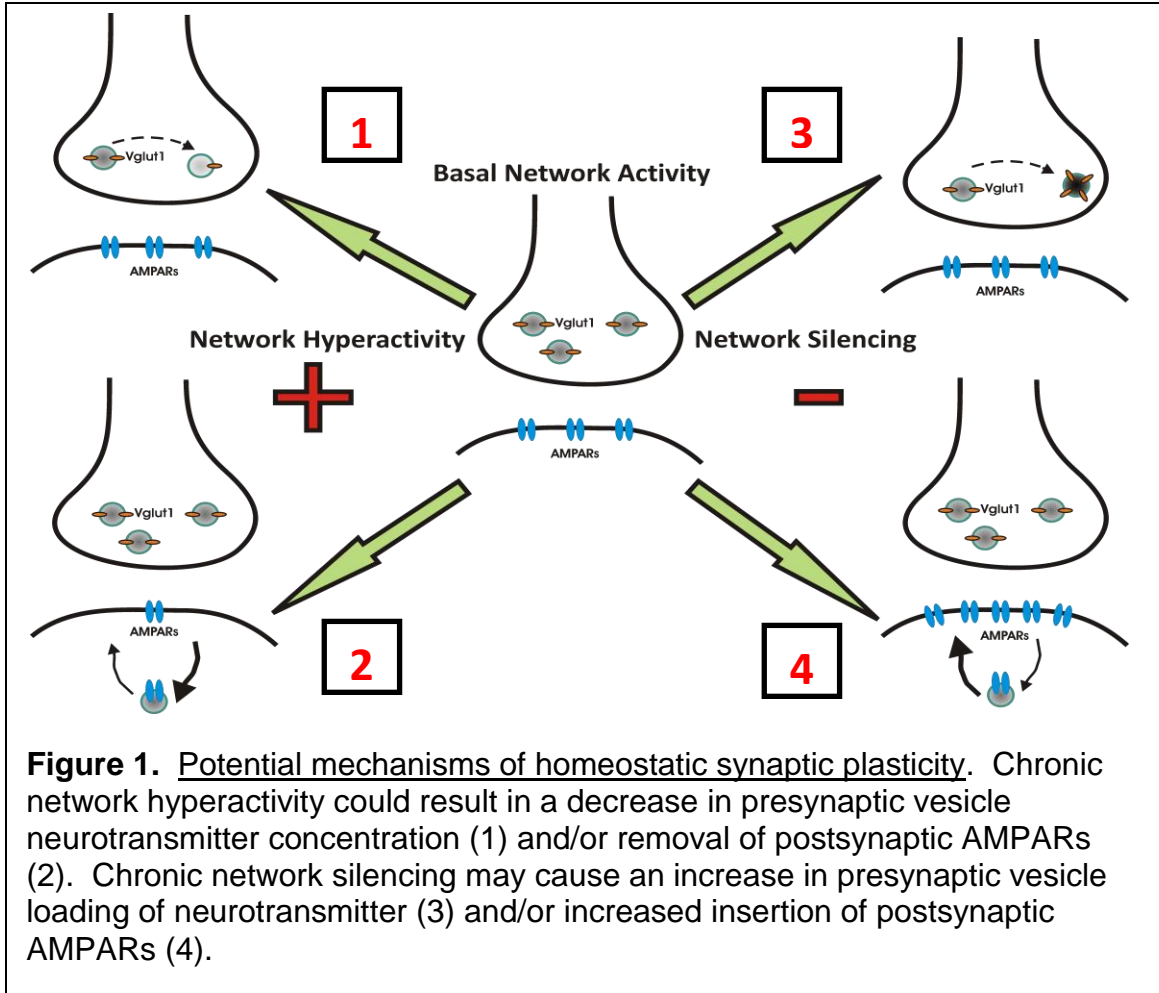
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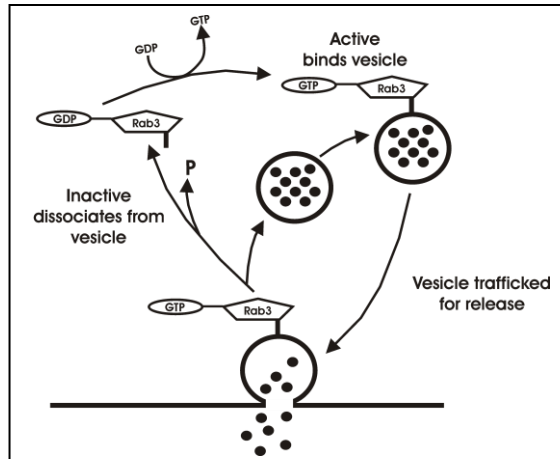
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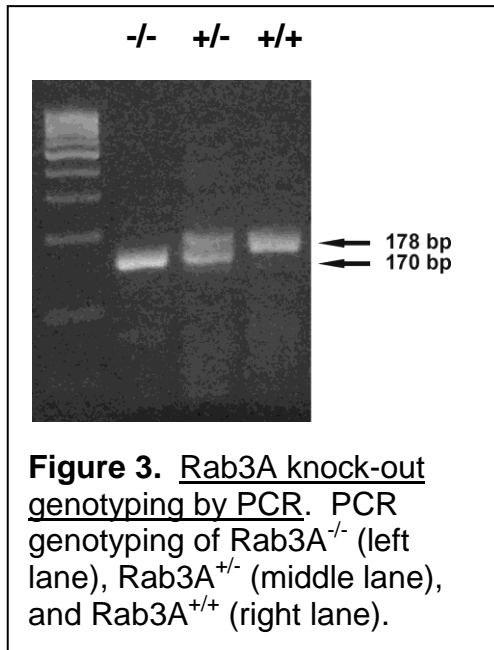


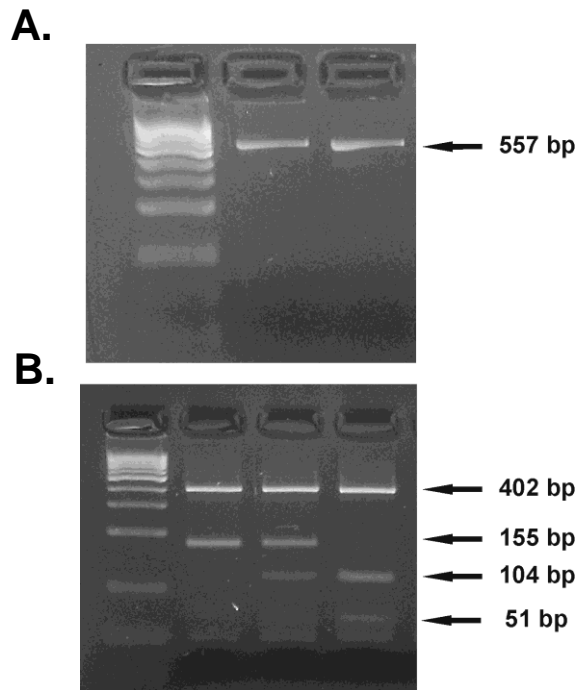
**Figure 1.** Potential mechanisms of homeostatic synaptic plasticity. Chronic network hyperactivity could result in a decrease in presynaptic vesicle neurotransmitter concentration (1) and/or removal of postsynaptic AMPARs (2). Chronic network silencing may cause an increase in presynaptic vesicle loading of neurotransmitter (3) and/or increased insertion of postsynaptic AMPARs (4).



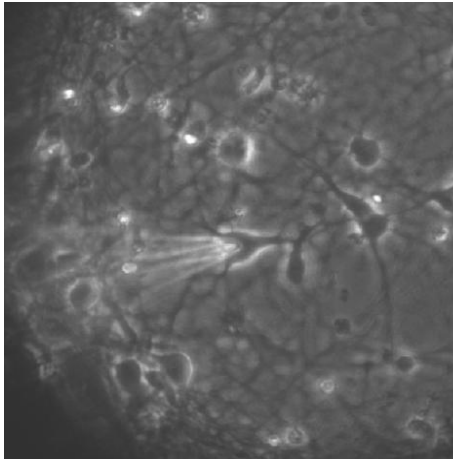
**Figure 2. The Rab GTPase cycle.**  
 The Rab GTPase cycles between its “on” GTP-bound form where it associates with synaptic vesicles targeting them to the cell membrane and its “off” GDP-bound form where it dissociates from vesicles.



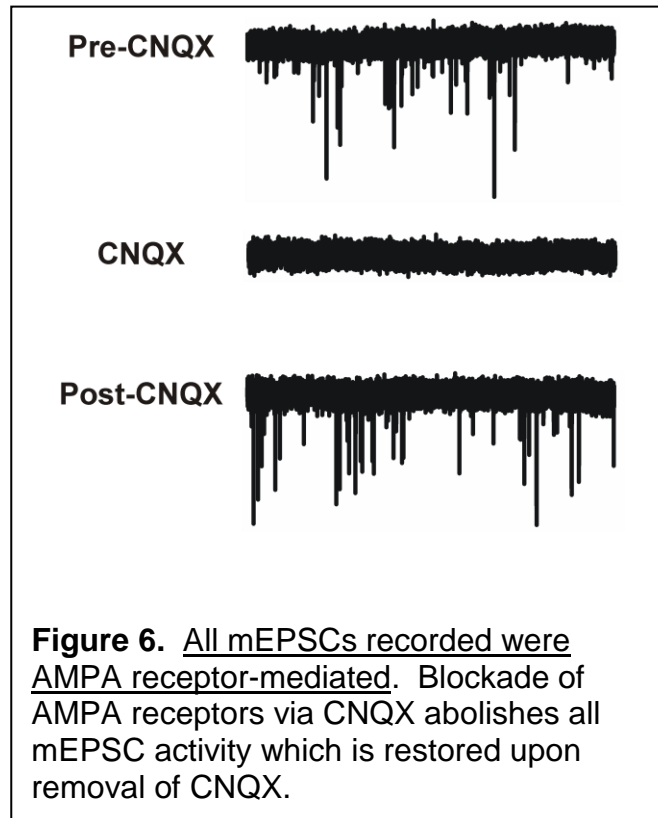


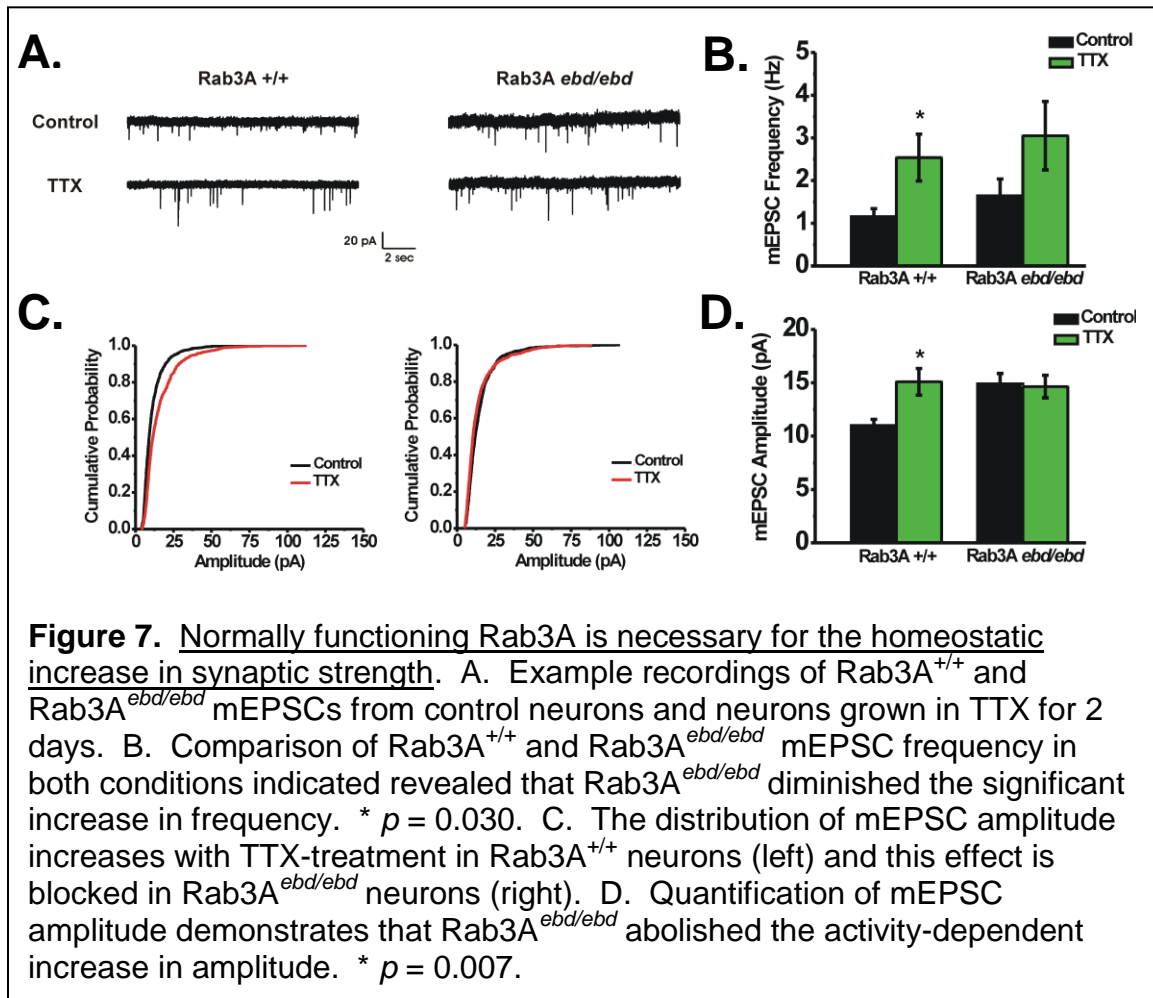


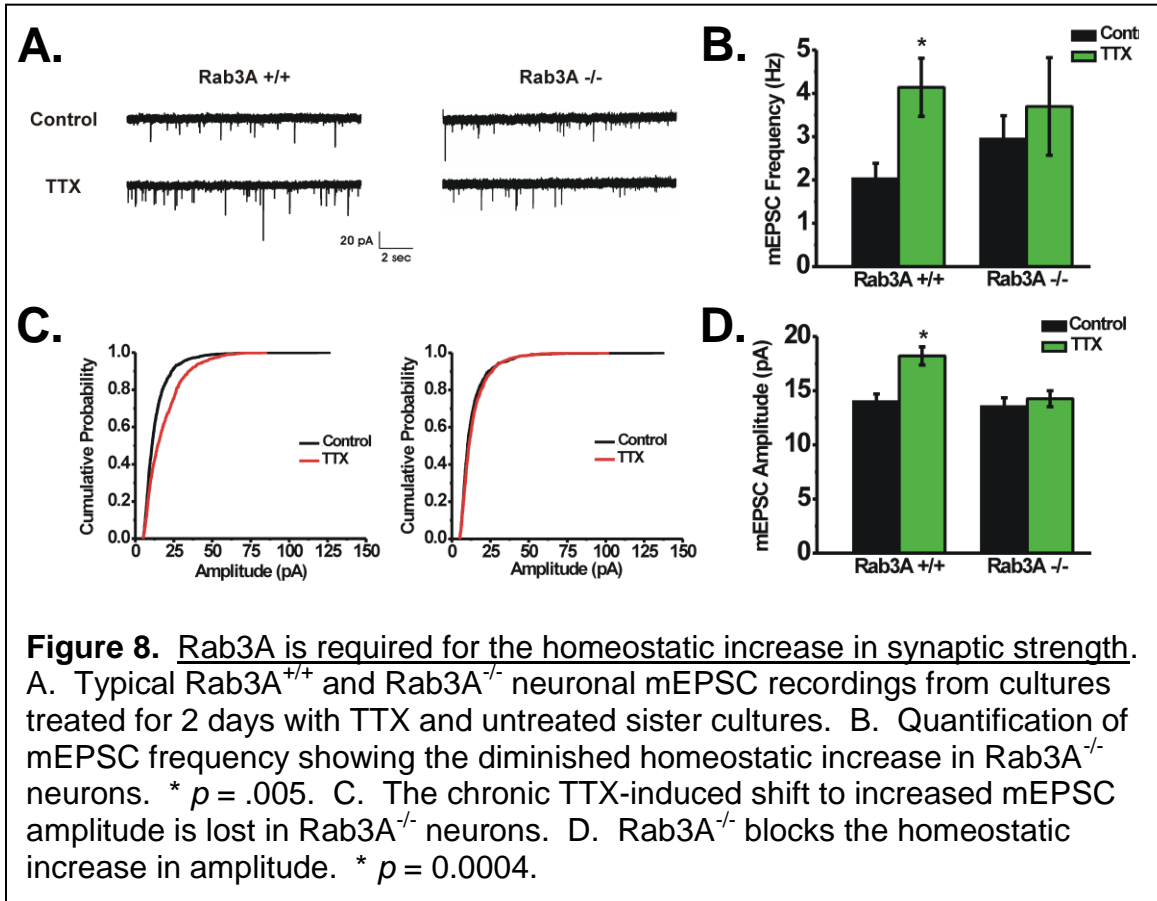
**Figure 4.** Rab3A *earlybird* genotyping. A. Rab3A *ebd*-targeted PCR. B. Restriction digest of Rab3A *ebd*-targeted PCR amplicons showing Rab3A<sup>+/+</sup> (left lane), Rab3A<sup>+/ebd</sup> (middle lane), and Rab3A<sup>ebd/ebd</sup> (right lane).

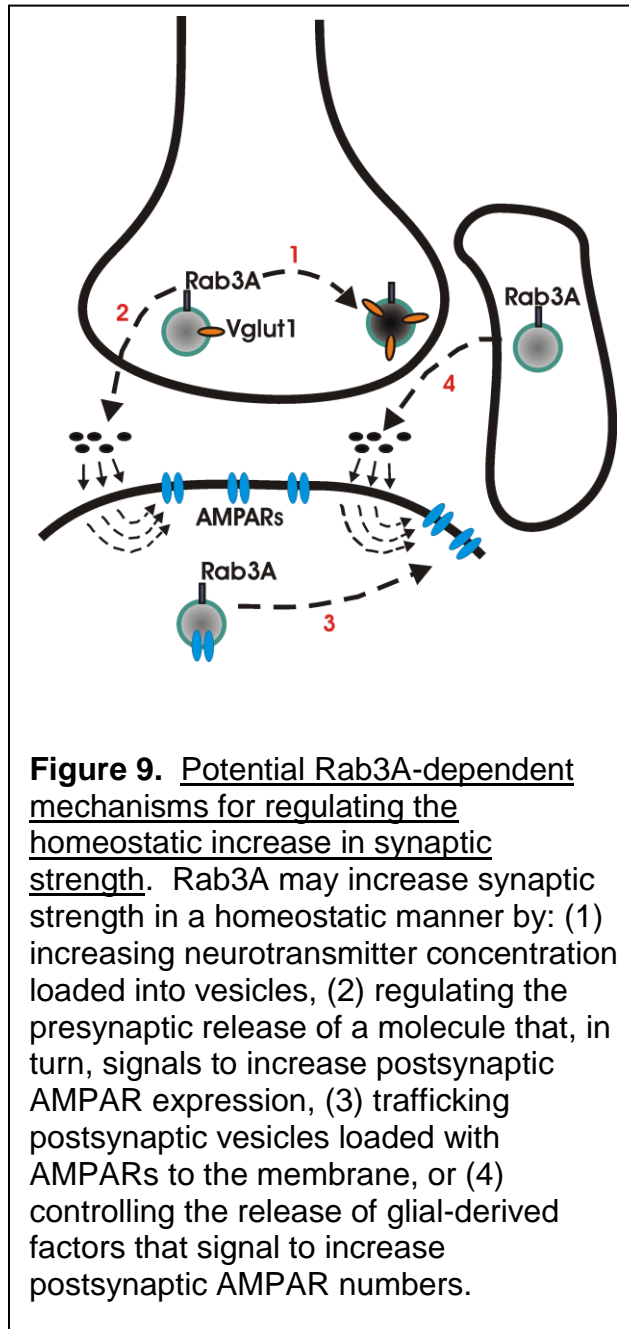


**Figure 5.** Patch electrode on a cultured cortical pyramidal neuron.

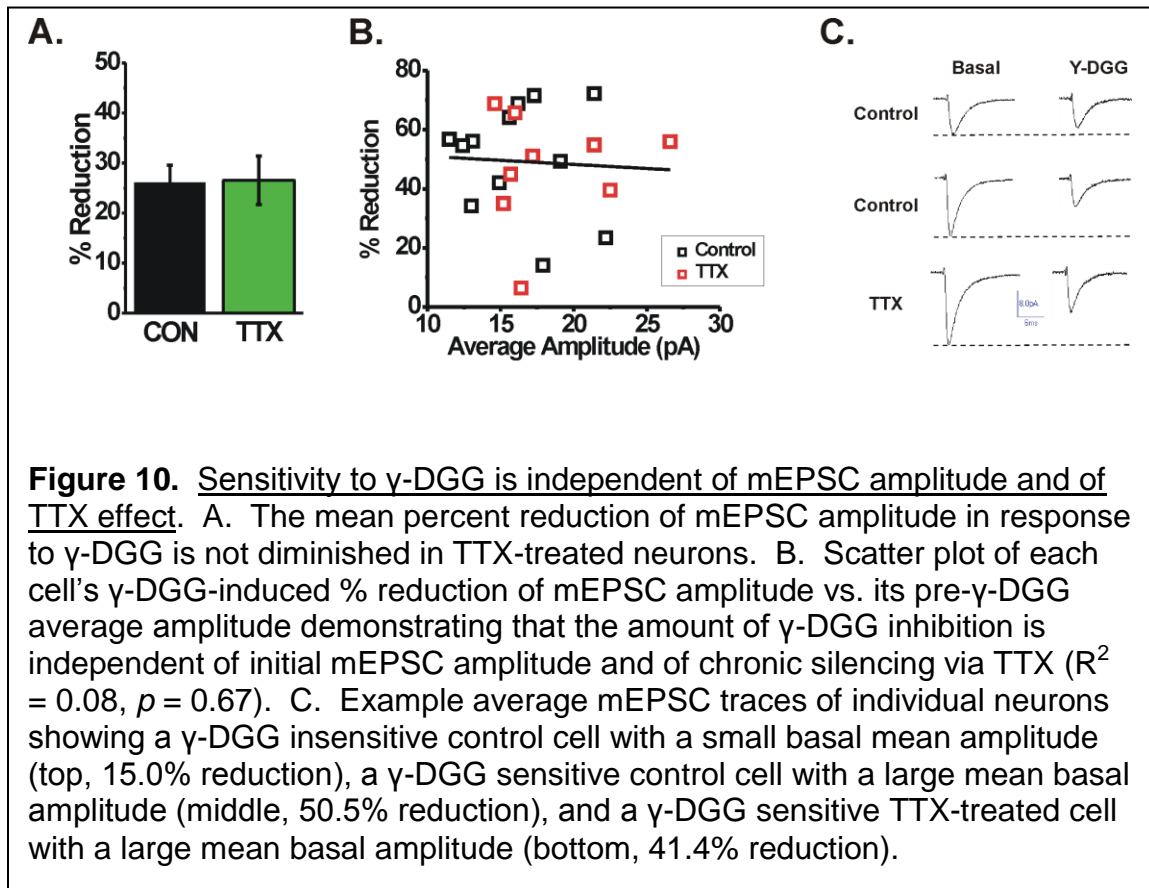




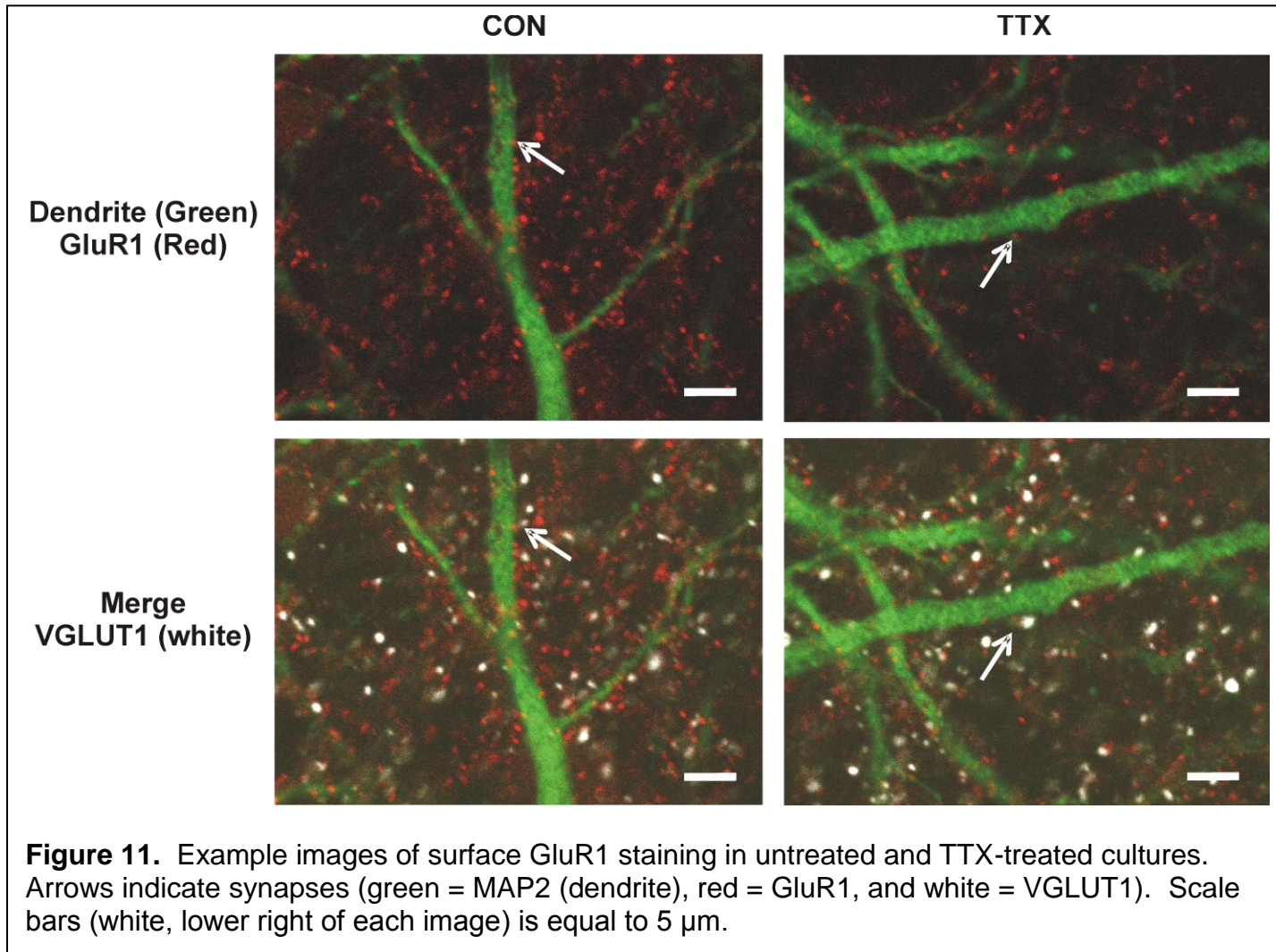


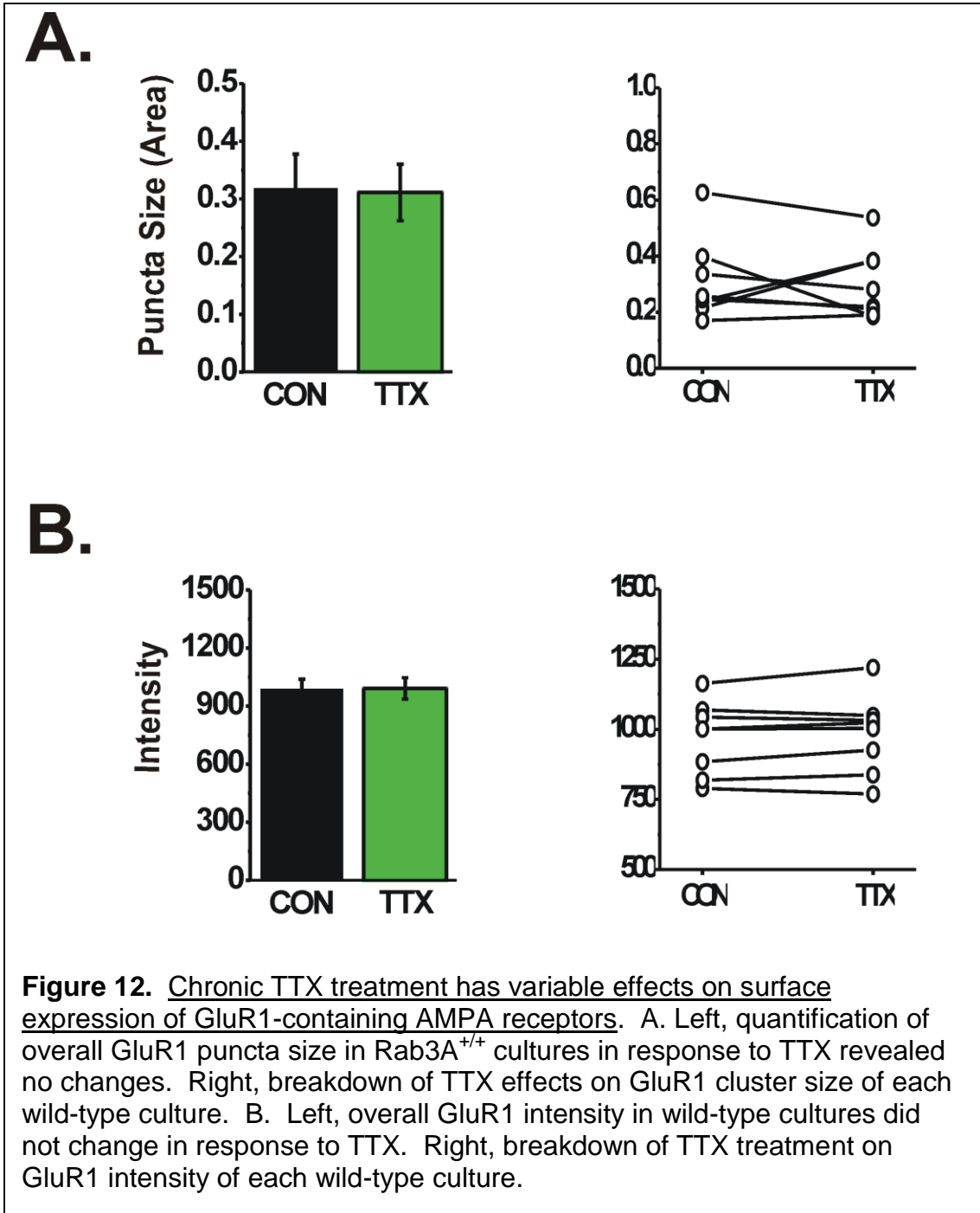


**Figure 9.** Potential Rab3A-dependent mechanisms for regulating the homeostatic increase in synaptic strength. Rab3A may increase synaptic strength in a homeostatic manner by: (1) increasing neurotransmitter concentration loaded into vesicles, (2) regulating the presynaptic release of a molecule that, in turn, signals to increase postsynaptic AMPAR expression, (3) trafficking postsynaptic vesicles loaded with AMPARs to the membrane, or (4) controlling the release of glial-derived factors that signal to increase postsynaptic AMPAR numbers.



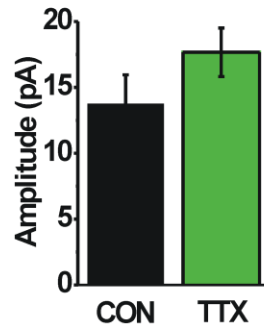






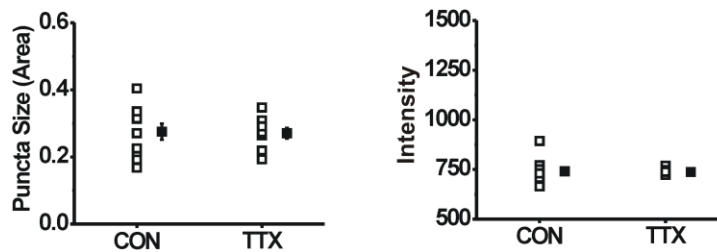
## Experiment 1

A.



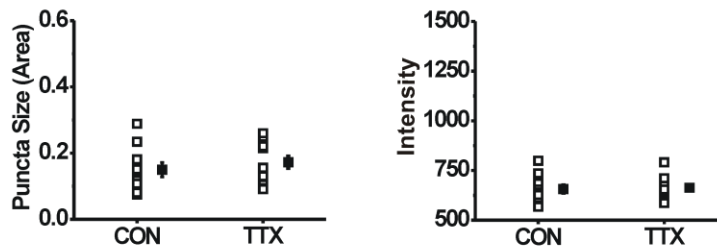
B.

Total GluR1 Staining



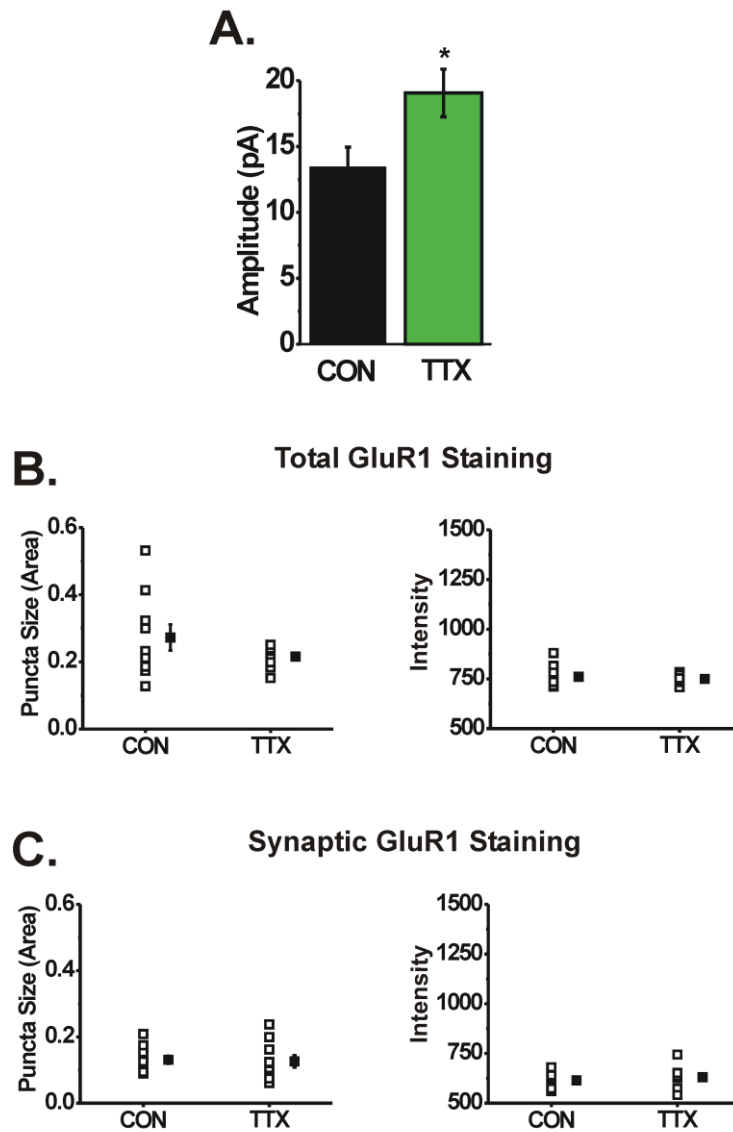
C.

Synaptic GluR1 Staining

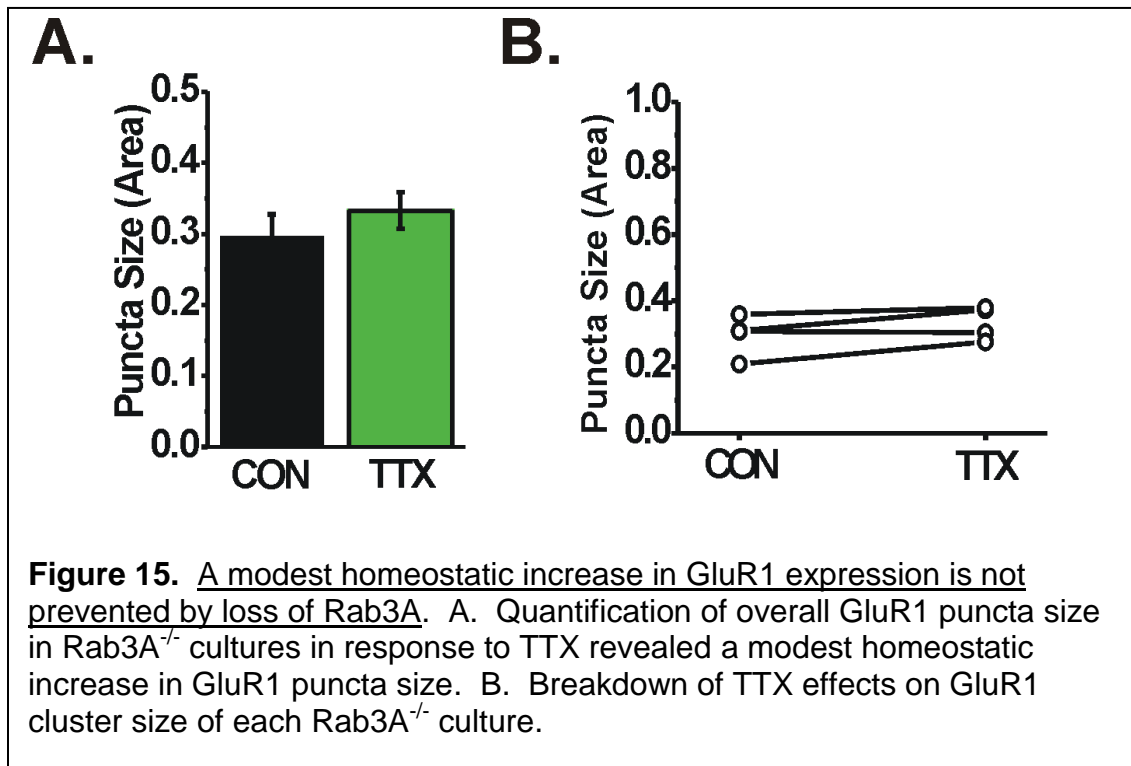


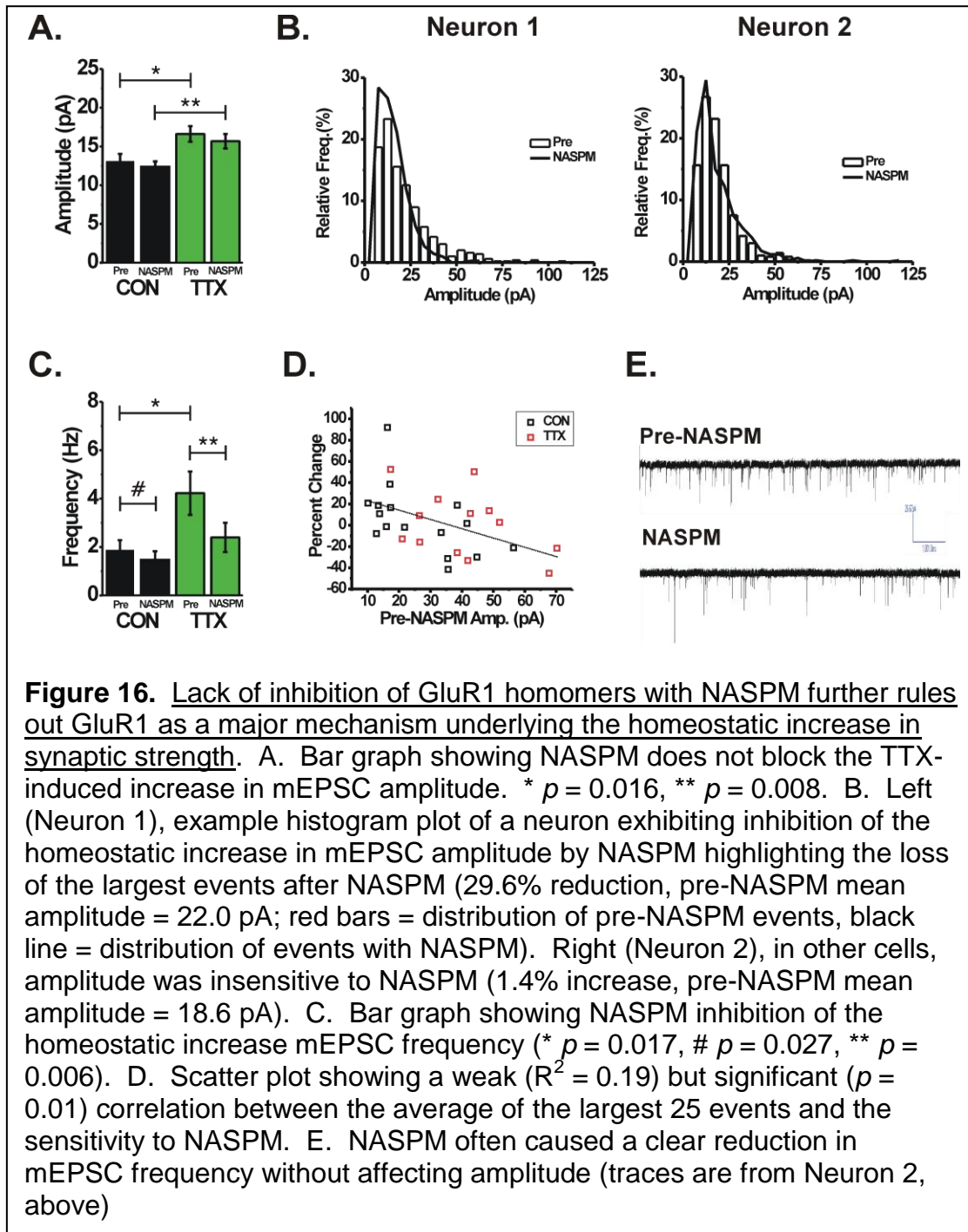
**Figure 13.** Individual experiment demonstrating a homeostatic increase in a culture's mEPSC amplitude is not accompanied by an increase in that culture's surface GluR1 expression. A. TTX-induced increase in culture mEPSC amplitude. Scatter plots of each dendrite's GluR1 staining (open squares) and their mean staining (filled squares) showing no change in B. total GluR1 puncta size (left) or intensity (right), or C. synaptic GluR1 puncta size (left) or intensity (right).

## Experiment 2

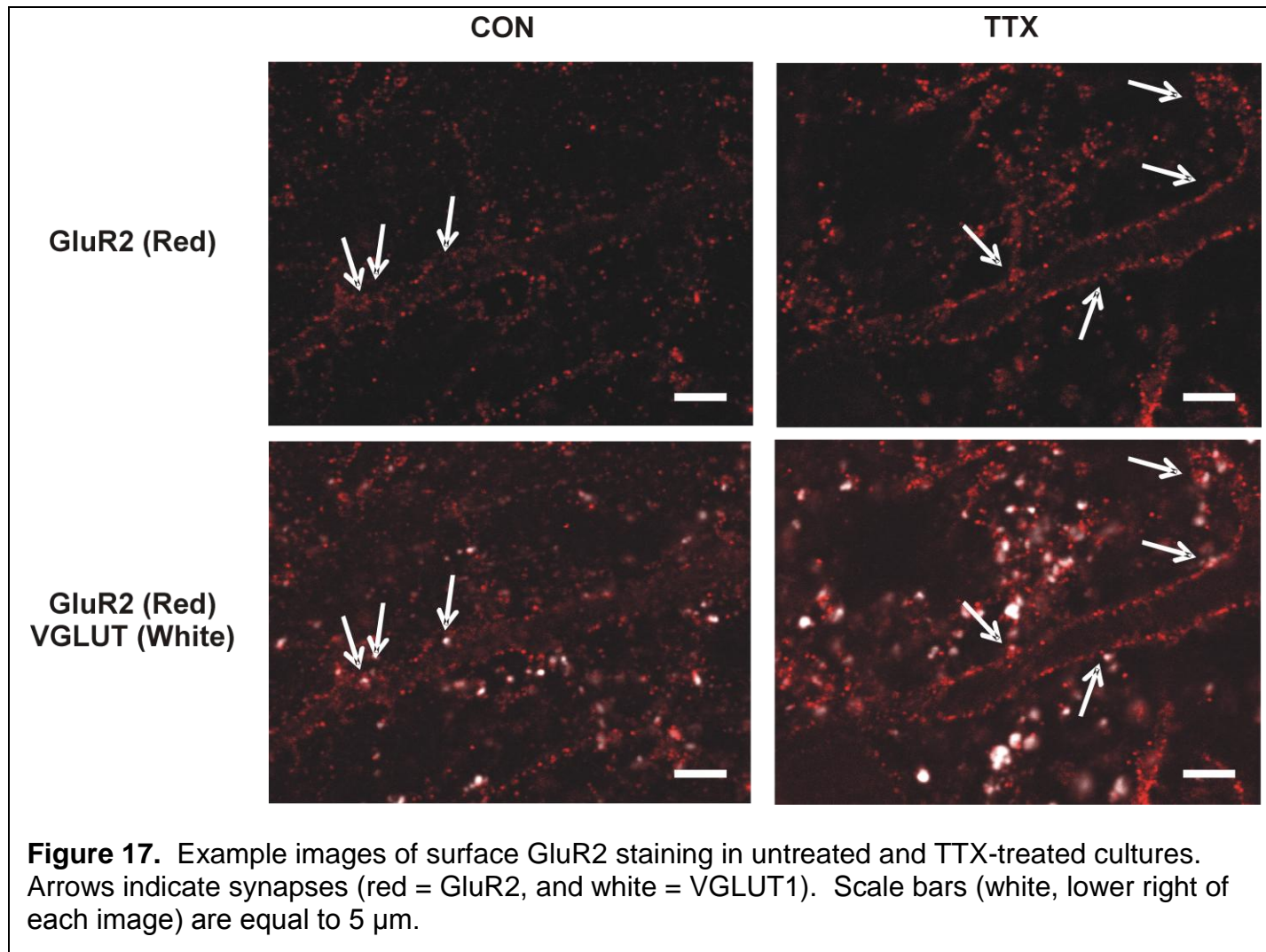


**Figure 14.** A second experiment demonstrating the same result as in Figure 13 – Despite a homeostatic increase in a culture’s mEPSC amplitude, there is no associated increase in that culture’s surface GluR1 expression. A. TTX-induced increase in culture mEPSC amplitude. Scatter plots of each dendrite’s GluR1 staining (open squares) and their mean staining (filled squares) showing no change in B. total GluR1 puncta size (left) or intensity (right), or C. synaptic GluR1 puncta size (left) or intensity (right).

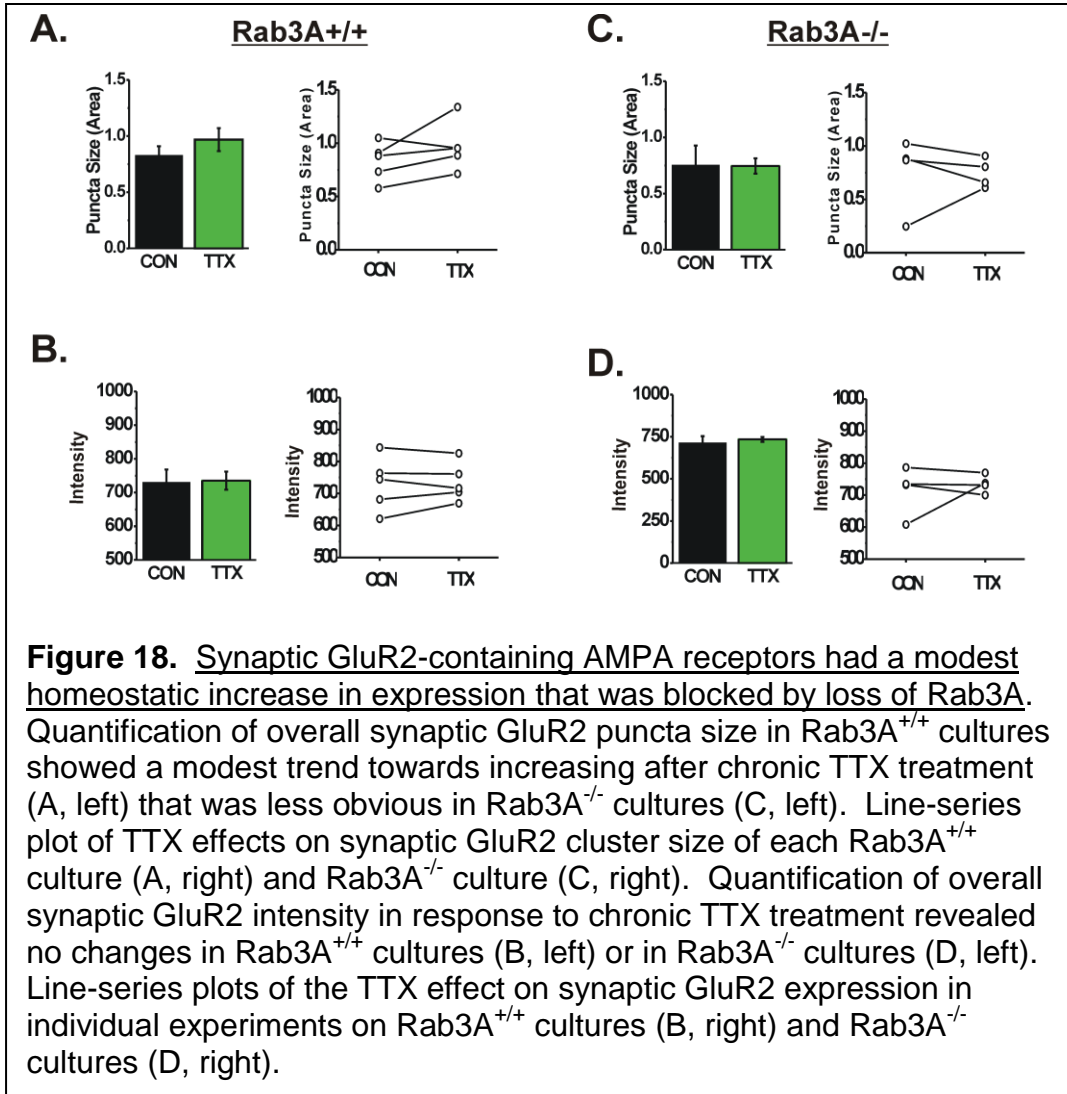




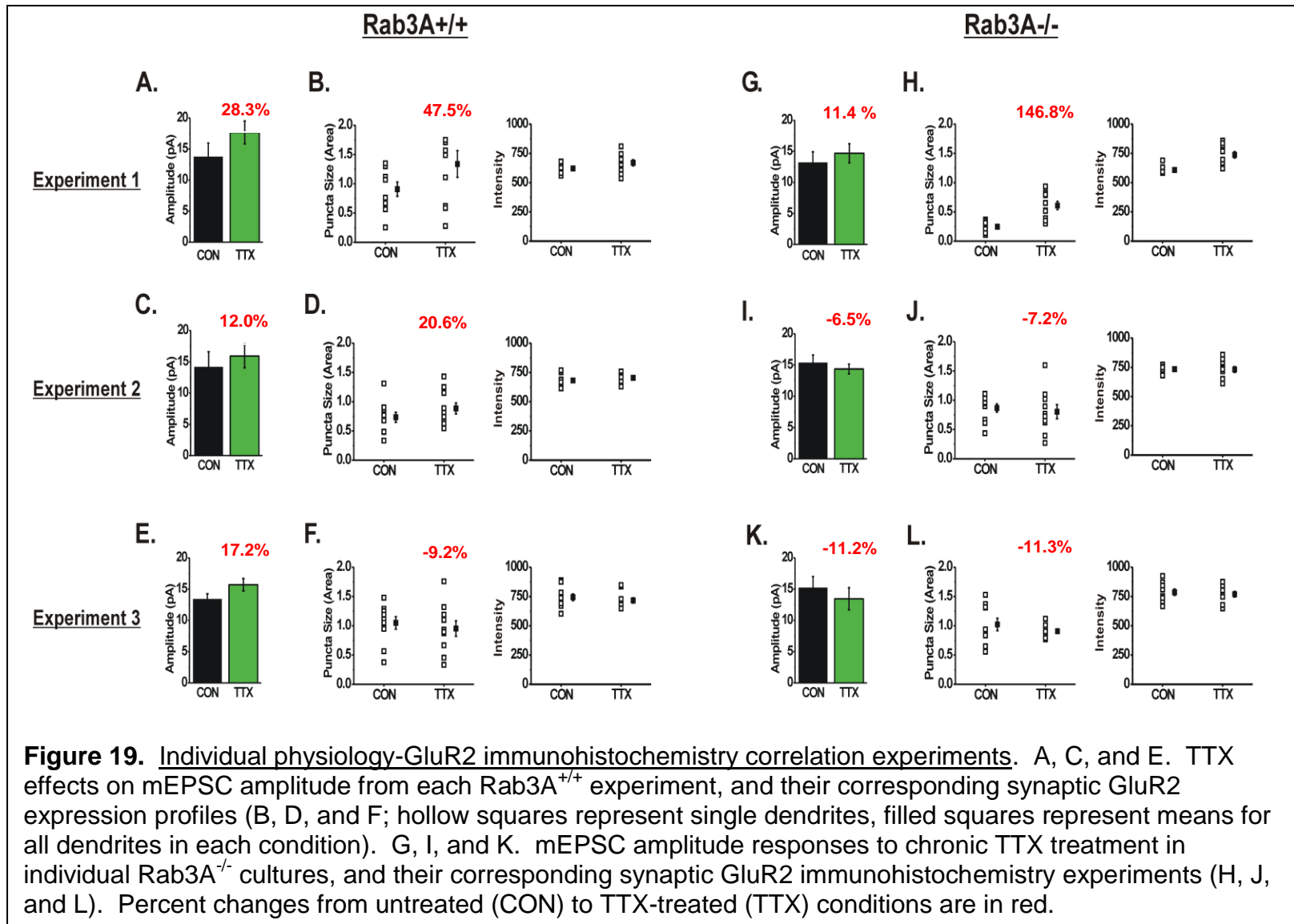
**Figure 16.** Lack of inhibition of GluR1 homomers with NASPM further rules out GluR1 as a major mechanism underlying the homeostatic increase in synaptic strength. A. Bar graph showing NASPM does not block the TTX-induced increase in mEPSC amplitude. \*  $p = 0.016$ , \*\*  $p = 0.008$ . B. Left (Neuron 1), example histogram plot of a neuron exhibiting inhibition of the homeostatic increase in mEPSC amplitude by NASPM highlighting the loss of the largest events after NASPM (29.6% reduction, pre-NASPM mean amplitude = 22.0 pA; red bars = distribution of pre-NASPM events, black line = distribution of events with NASPM). Right (Neuron 2), in other cells, amplitude was insensitive to NASPM (1.4% increase, pre-NASPM mean amplitude = 18.6 pA). C. Bar graph showing NASPM inhibition of the homeostatic increase mEPSC frequency (\*  $p = 0.017$ , #  $p = 0.027$ , \*\*  $p = 0.006$ ). D. Scatter plot showing a weak ( $R^2 = 0.19$ ) but significant ( $p = 0.01$ ) correlation between the average of the largest 25 events and the sensitivity to NASPM. E. NASPM often caused a clear reduction in mEPSC frequency without affecting amplitude (traces are from Neuron 2, above)

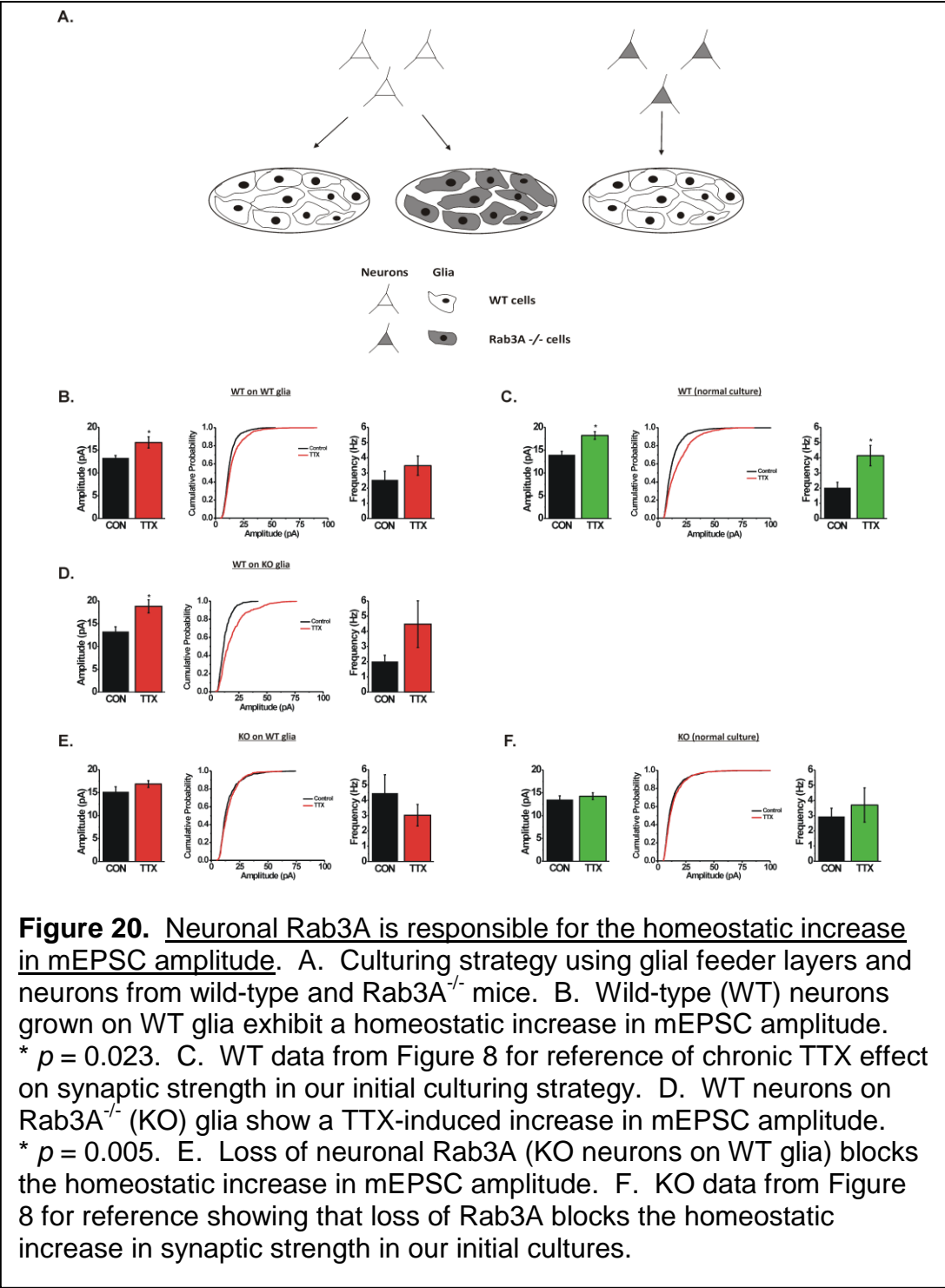






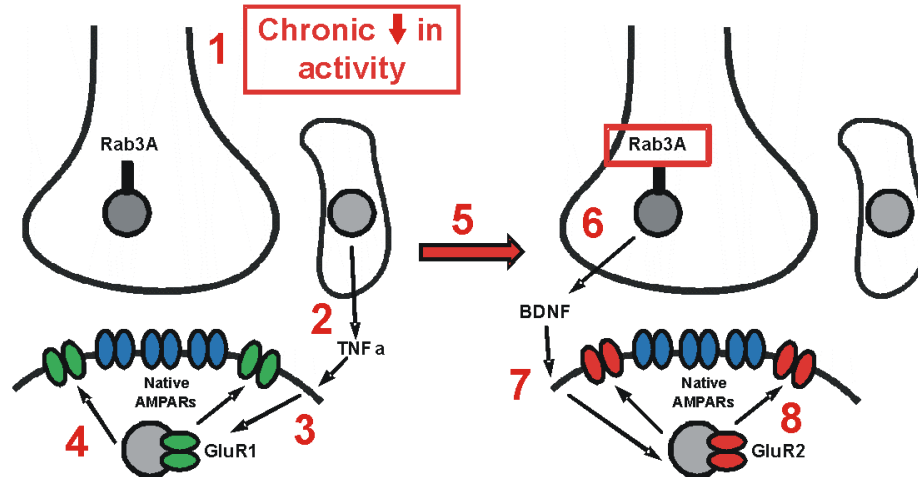






**Phase 1: Induction**  
(~ Day 1)

**Phase 2: Maintenance**  
(~ Day 2)



**Figure 21.** Rab3A-dependent model of a biphasic mechanism for the homeostatic increase in synaptic strength. Phase 1, or the Induction Phase (~ day 1), is initiated by a chronic decrease in network activity (1) triggering glial release of TNF $\alpha$  (2) that signals to the postsynaptic neuron (3) to increase GluR1 incorporation into the membrane (4). In the transition from day 1 to day 2 after activity blockade (5), Phase 2, or the Maintenance Phase, is initiated by triggering Rab3A-dependent release of BDNF from the presynaptic neuron (6), which travels across the synaptic cleft activating a signaling cascade in the postsynaptic neuron (7) to replace GluR1 receptors and increase GluR2 levels (8).

Article	Species	Age	Tissue	Prep	DIV	Type of Block	Length of Block	Mechanism	Method Quantified
Aoto et al., 2008	rat	E22	Hippo	Dissociated	14-15	TTX	24 hrs	GluR1	
Soden & Chen, 2010	mouse	P0-P1	Hippo	Dissociated	~14	TTX	24 hrs	GluR1	Western
Shepherd et al., 2006	rat	E18-E19	Hippo Cortical	Dissociated	12-14	TTX	48 hrs	GluR1	Intensity Western
Correa et al., 2012	mouse	P0-P1	Hippo	Dissociated	15-18	TTX	24 hrs	GluR1	Intensity
Thiagarajan et al., 2005	rat	P2-P4	Hippo	Dissociated	16-17	receptor	24 hrs	GluR1	Western
Gong et al., 2007	mouse	P0	Cortical	Dissociated	14-17	receptor	24 hrs	GluR1	Western
Ju et al., 2004	rat	E18	Hippo	Dissociated	5-8	TTX	24 hrs	GluR1	Synaptic
Stellwagen & Malenka, 2006	mouse	P0	Hippo	Dissociated	13-15	TTX	48 hrs	GluR1	Area
Hou et al., 2008	rat	E18	Hippo	Dissociated	~14	Kir2.1	24 hrs	GluR1	Intensity
Goel et al., 2006	rat	5 wks	Visual cortex	Slice	na	Dark rearing	1 wk	GluR1	Western
Goel et al., 2011	mouse	P21	Visual cortex	Slice	na	Dark exposure	24 hrs	GluR1	Western
Sun & Wolf, 2009	rat	P1	NAc/PFC co-culture	Dissociated	2-3 wks	receptor	24-72 hrs	GluR1 & GluR2	Surface Area Surface # Synaptic Nonsynaptic Cellular
Wierenga et al., 2005	rat	P3-P4	Cortical	Dissociated	7-10	TTX	48 hrs	GluR1 & GluR2	Intensity Synaptic Nonsynaptic
Cingolani et al., 2008	rat	P0	Hippo	Dissociated	9-15	TTX	48 hrs	GluR2	Relative fluorescence
Gainey et al., 2009	rat	P2-P4	Cortex	Dissociated	6-10	TTX	24 hrs	GluR2	Pharmacology

**Table 1.** Summary of studies examining the involvement of AMPA receptors in homeostatic synaptic plasticity.

1° Antibody	Labels	Species	Conc.	Supplier	2° Antibody	Reactivity	Conc.	Supplier
anti-MAP2 (mono)	Soma & dendrites	Mouse	1:300	Sigma	488	anti-mouse	1:225	Jackson
anti-VGLUT1	Presynaptic terminals	Guinea pig	1:4000	Chemicon	Cy3	anti-guinea pig	1:225	Jackson
anti-GluR1	GluR1 receptor	Rabbit	1:10	EMD Millipore	Cy5 (649)	anti-rabbit	1:225	Jackson
Anti-MAP2	Soma & dendrites	Chick	1:2500	Abcam	488	Anti-chick	1:225	Jackson
anti-VGLUT1	Presynaptic terminals	Rabbit	1:4000	Synaptic Systems	Cy5	anti-rabbit	1:225	Jackson
anti-GluR2	GluR2 receptor	Mouse	1:40	EMD Millipore	CY3	Anti-mouse	1:225	Jackson

**Table 2.** List of antibodies used for immunohistochemistry experiments. GluR1 and GluR2 expression were examined in separate experiments.