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Mechanisms Mediating Adaptive Presynaptic Muting Induction

by

Devon Christine Crawford

A dissertation presented to the
Graduate School of Arts and Sciences
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

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2012

ABSTRACT OF THE DISSERTATION

Mechanisms mediating adaptive presynaptic muting induction

by

Devon Christine Crawford

Doctor of Philosophy in Neurosciences

Washington University in St. Louis, 2012

Professor Steven Mennerick, Chairperson

Neurons are responsible for information processing within the nervous system, so strong perturbations of neuronal function have far-reaching consequences within the neural network. Damage in response to excess excitation, as occurs during stroke or seizure, is known as excitotoxicity. One method utilized by neurons for reducing excitotoxicity within an overly activated neuronal network is to arrest excitatory neurotransmitter release from presynaptic terminals. The mechanisms responsible for inducing this presynaptic silencing (or “muting”), however, have been elusive. In order to elucidate the signals responsible, I used molecular techniques in defined networks of cultured neurons from the mammalian hippocampus, a well-studied brain region known to be important for learning and memory but susceptible to excitotoxic damage.

Calcium serves as a signal transducer during excitotoxicity and many forms of synaptic plasticity, but the signaling cascades in presynaptic silencing were previously unknown. In neurons individually depolarized via heterologous ion channel activation, I showed that calcium influx led to cell death while channel expression led to synaptic depression, although muting was not confirmed. Calcium, however, was not necessary

for presynaptic muting after strong depolarization. Instead, inhibitory G-protein signaling induced silencing through cyclic adenosine monophosphate (cAMP) reduction but surprisingly not via activation of likely candidate receptors. This cAMP reduction contributed to loss of proteins important for vesicle fusion at the presynaptic terminal.

I also found that astrocytes, support cells in the nervous system that have garnered attention recently for their ability to modulate neuronal function, were required for the proper development of presynaptic muting in hippocampal neurons. Soluble factors released by astrocytes were permissive, but not instructive, for silencing induction. Thrombospondins were identified as the astrocyte-derived factors responsible for muting competence in neurons, and they act through binding to the $\alpha 2\delta$ -1 subunit of voltage-gated calcium channels. cAMP-activated protein kinase A exhibited dysfunctional behavior in the absence of thrombospondins, potentially explaining the presynaptic muting deficit in an astrocyte-deficient environment.

Together these results clarify the molecular mechanisms responsible for an underappreciated form of neuroprotective synaptic plasticity and provide potential therapeutic targets for a number of disorders expressing excitotoxic damage.

Acknowledgments

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I am also grateful for the mentorship I have received from my Ph.D. advisor, Dr. Steven Mennerick. He is a careful scientist and patient teacher, and hopefully my conspiracy to nominate him for his recently received Outstanding Faculty Mentor Award has illustrated my appreciation for the time he spends mentoring his students. Without his firm guidance during the rough research spots and flexibility during times when I asserted independence, I would not be as successful or feel nearly as prepared to embark on the next chapter in my career. He has been a steadfast cheerleader for my work and accomplishments. I also appreciate all of the advice and support I have received over the years from my thesis committee members. From proposing clever experiments to showing genuine enthusiasm for my work, they have contributed much to my project.

I would be remiss if I did not thank all past and present members of the Mennerick lab. Ann Benz and Amanda Taylor have fought valiantly against the “malevolent deity” to keep our cell cultures in good health, and they also keep the lab in

good spirits with baked goods. I owe Krista Moulder and Xiaoping Jiang gratitude for their early work on presynaptic muting that laid the groundwork for my project and all lab group members, including Charles Zorumski, Tina Chang, Joshua Hogins, Gerry Kress, Christine Wroge, Andrea Lui, John Cirrito, Jenny Shu, Bill Dribben, Pato Rojas, Catherine Wasser, Andrew Linsenhardt, Larry Eisenman, Yukitoshi Izumi, Kazuhiro Tokuda, and Liu Lin Thio, for advice, discussion, and helpful feedback during lab meetings. I especially want to thank Mariangela Chisari for her willingness to take time out of her day to help me with anything I needed and Julian Meeks for his continued advice and feedback despite having left the lab when I was a mere rotation student. Also, I will fondly remember late evening discussions with honorary lab member Ariel Lyons-Warren—someday I may learn how to take your advice to stop working and go home.

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Last, but certainly not least, I thank my family: my parents who have never doubted that I could accomplish anything I desire (and jokingly told me to do whatever I wanted with my life as long as I got a Ph.D. in it) and my brother and extended family who are forever loyal and supportive. Finally, I thank my other half, Timothy Sauline, for going on this journey with me and being patient with me when the lab came first. He has been the rock that has kept me steady through the good times and the bad.

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List of abbreviations

AGS: activator of G-protein signaling	LTD: long-term depression
AMPA: 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid	LTP: long-term potentiation
ATP: adenosine triphosphate	MG-132: carbobenzoxy-L-leucyl-L-leucyl-L-leucinal
BAPTA: bis(2-aminophenoxy)ethane tetraacetic acid	mGluR: metabotropic glutamate receptor
BDNF: brain-derived neurotrophic factor	NBQX: 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide
cAMP: cyclic adenosine monophosphate	NMDA: N-methyl-D-aspartic acid
CCPA: 2-chloro-N ⁶ -cyclopentyladenosine	PKA: protein kinase A
CDK5: cyclin-dependent kinase 5	p_r: probability of vesicle release
CREB: cAMP response element binding protein	Syn-YFP: synaptophysin-YFP
D-APV: D-(-)-2-amino-5-phosphonopentanoic acid	TNFα: tumor necrosis factor alpha
DIV: day <i>in vitro</i>	TRPM8: transient receptor potential cation channel, subfamily M, member 8
EGTA: ethylene glycol-bis (β -aminoethyl ether)-N, N, N', N'-tetraacetic acid	TRPV1: transient receptor potential vanilloid receptor 1
EPSC: excitatory postsynaptic current	TSP: thrombospondin/thrombin-sensitive protein
G-protein: guanine nucleotide-binding protein	TTX: tetrodotoxin
GABA: γ -aminobutyric acid	UPS: ubiquitin-proteasome system
GPCR: G-protein-coupled receptor	vGluT-1: vesicular glutamate transporter 1
IPSC: inhibitory postsynaptic current	YFP: yellow fluorescent protein

Chapter 1

Introduction and thesis objectives

This chapter contains text excerpts and figures (Figures 1, 2, and 4) from a previously published manuscript:

Crawford DC, Mennerick S (2012). Presynaptically silent synapses: dormancy and awakening of presynaptic vesicle release. *Neuroscientist* 18(3): 216.

Author contributions for the citation above:

D.C.C. wrote the paper and made the figures. S.M. edited the manuscript.

Adaptive presynaptic muting

Neurons transmit electrical signals as the basis of information processing in the nervous system. Chemical synapses mediate much of the communication between neurons. Synapses pivotally influence the flow of information throughout the neural network and are primary sites of malleability that may underlie behavioral alterations and memory formation. Modulation of synaptic strength takes many forms. One form includes mature, functioning synapses rendered dormant, but the mechanisms underlying this are poorly understood. These “silent” synapses are deficient either in neurotransmitter release or in postsynaptic receptors (Fig. 1). Here I will focus on presynaptically silent synapses, as postsynaptically silent synapses have been discussed extensively elsewhere and are not the focus of this dissertation (Kerchner and Nicoll, 2008).

Presynaptically silent synapses are identified throughout the literature also as “dormant,” “mute,” “non-functional,” or “inactive” presynaptic terminals. They are characterized by terminals that fail to release transmitter in response to strong calcium influx and are found in a variety of species and neurotransmitter systems. Dormant presynapses are distinct from weakly transmitting terminals because they are release-incompetent even after accounting for the normal heterogeneity of vesicle release probability at presynaptic terminals. Dormancy, therefore, represents a qualitative change in release competence. It remains unclear how the absence of vesicle fusion at strategic synaptic locations alters signaling within a network, but this phenomenon may play important roles in information processing and in pathology.

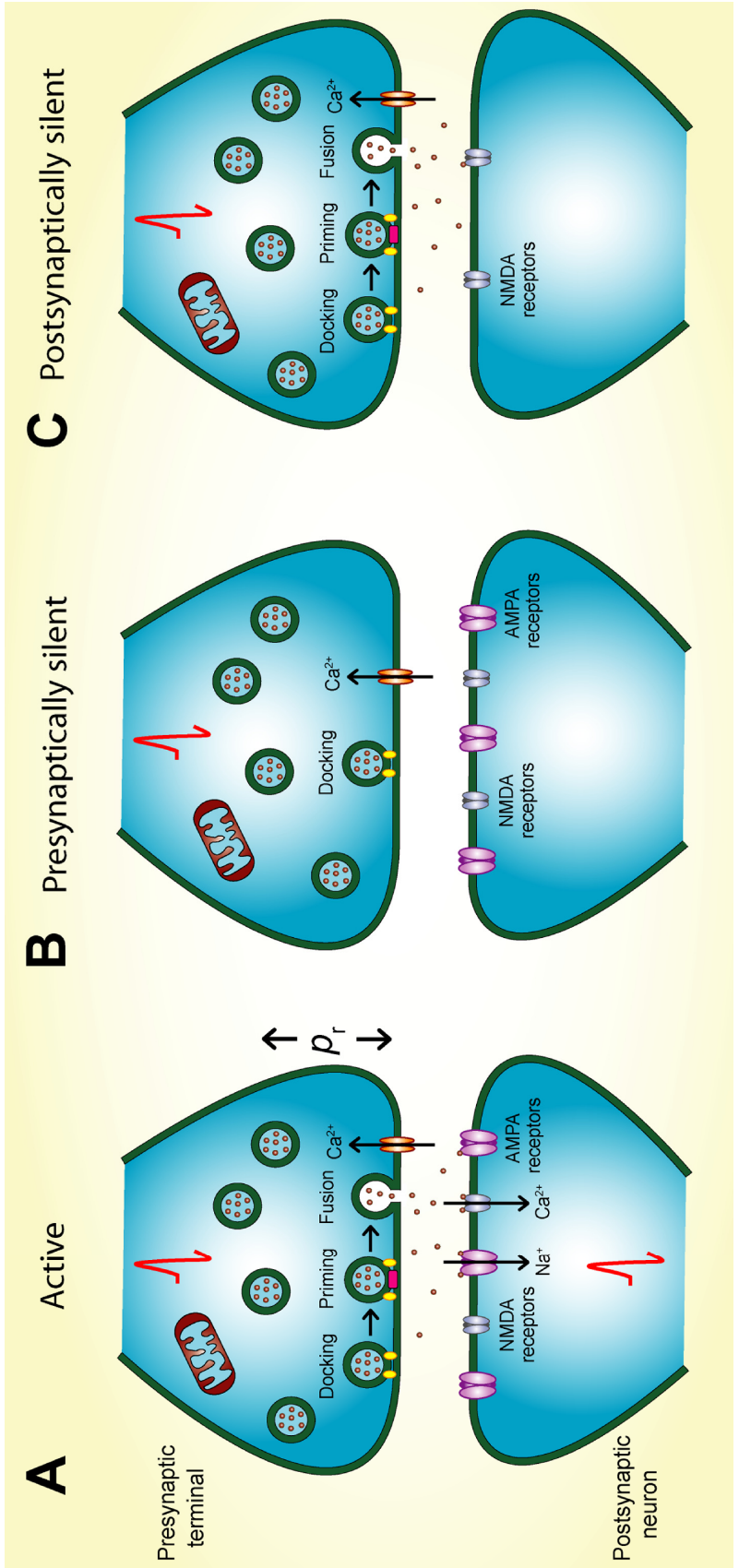


Figure 1. Categories of silent synapses. **A.** Active synapses consist of presynaptic terminals with functional vesicle docking, priming, and probabilistic release upon calcium influx, all powered primarily by mitochondrial ATP production. The probability of vesicle release (p_r) is modulated without altering the qualitative release competence of the terminal. Neurotransmitter released from active presynaptic terminals binds to postsynaptic receptors and causes a postsynaptic response. At glutamate synapses, for example, glutamate released via presynaptic vesicle fusion will bind to AMPA receptors, allowing net cation influx that directly depolarizes the target cell and relieves voltage-dependent magnesium block of NMDA receptors (not depicted). Depolarizing effects of activated AMPA and NMDA receptors contribute to action potential generation. **B.** In presynaptically silent synapses, vesicle docking is intact, but priming and fusion are impaired, even with strong depolarization and calcium influx that overcomes low vesicle release probability. Without transmitter release, there is no postsynaptic response. **C.** Postsynaptically silent synapses maintain active presynaptic terminals, but the postsynaptic membrane is missing receptors necessary to generate a response. At glutamate synapses, AMPA receptors are absent, leaving NMDA receptors unable to overcome voltage-dependent block.

Evidence for presynaptic muting:

Early evidence for presynaptic silencing, or muting, came from studies using quantal analysis to calculate the number of release sites for neurotransmitter. By analyzing the underlying statistical structure of transmitter release, quantal analysis describes the size of postsynaptic current due to fusion of a single vesicle (quantal size, q), the number of functional release sites (n), and the probability that an action potential will cause vesicle fusion (probability of vesicle release, p_r) (Del Castillo and Katz, 1954; Redman, 1990). These calculations allow for inferences about the function of a synaptic connection. Using quantal analysis in multiple systems, including crayfish and rodent neurons, studies have demonstrated that quantal n is smaller than the number of physical synaptic connections between neurons (Neale et al., 1983; Wojtowicz et al., 1991). In other words, there are typically some non-functional synaptic connections present.

Strong evidence for a presynaptic locus of dormancy came from studies of the Mauthner cell, which functions in an auditory escape response pathway in goldfish. Membrane potential changes are reliably transmitted from stimulated presynaptic fibers to the lateral dendrite of the Mauthner cell through electrotonic coupling via gap junctions. Some presynaptic fibers, however, fail to transmit information through parallel chemical synapses (Faber et al., 1991). The maintained amplitude of the electrotonic potential argues against action potential conduction failure as an explanation for synaptic inefficacy between some neuron pairs. A small minority of the chemical synapses, however, can be activated by loading the presynaptic neuron with a cesium-containing solution (Faber et al., 1991). This manipulation blocks potassium channels, which broadens the action potential and provides prolonged depolarization to the presynaptic

terminal. The ability to awaken the synapse with a presynaptic manipulation suggests a presynaptic deficit but a full complement of postsynaptic receptors. Although the awakened terminals could simply have low vesicle release probability, the synapses that could not be awakened may represent truly dormant terminals. This discovery prompted studies of the induction and expression mechanisms for silent synaptic connections.

More recently, fluorescence visualization of presynaptic function has allowed the direct study of presynaptic muting without ambiguities introduced by calculating n from statistical models. Styryl dyes like FM1-43 partition into extracellularly-exposed membrane to reveal recycling vesicles. The number of active terminals, labeled with FM1-43 staining, is smaller than the total number of synaptic varicosities in dissociated hippocampal neurons (Ma et al., 1999), supporting the findings from previous studies that not all structurally-defined synapses are active. Dormant presynaptic terminals are presently defined as those terminals that label positively for synaptic proteins but not with styryl dyes during strong stimulation that should empty all release-competent vesicles. For example, synapses from cultured neurons that immunolabel for synaptophysin, Piccolo, GABA_A receptors, or vesicular glutamate transporter 1 but not with FM1-43 are stereotypic examples of inactive presynaptic terminals (Kannenberget al., 1999; Altrock et al., 2003; Moulder et al., 2004; Fig. 2). Typically, these terminals have normal ultrastructure, including vesicle arrangement, and normal postsynaptic receptor function (Moulder et al., 2004; Moulder et al., 2006).

An increase in silent presynaptic terminals is registered in electrophysiological measures of excitatory postsynaptic currents as a decrease in the size of a neuron's readily releasable pool of vesicles rather than a decrease in the vesicle release probability

(Moulder et al., 2004). Dormant presynaptic terminals cannot be forced to release vesicles by strong stimulation or by secretagogues that elicit calcium-independent vesicle fusion. These findings suggest that dormant presynaptic terminals are structurally mature and intact but have a deficit in vesicle fusion that renders the synapse unable to function. Some evidence suggests that this deficit is at the level of vesicle priming (Moulder et al., 2006; Jiang et al., 2010), the maturation of release competence after vesicle docking but prior to calcium-dependent fusion. Thus, altered presynaptic priming protein function may induce dormancy.

Induction of presynaptic muting:

Silent presynaptic terminals are present in the absence of any manipulations, but the mechanisms controlling dormancy can be examined by manipulating the number of dormant terminals. In cultured hippocampal neurons, prolonged strong depolarization increases dormancy selectively in glutamatergic terminals (Fig. 2A, B), and this is slowly reversible (Moulder et al., 2004). Weaker depolarization and increased spiking over several days also increase dormancy in a tetrodotoxin-sensitive manner (Moulder et al., 2006). Hypoxic depolarization also induces dormancy (Hogins et al., 2011; also see Appendix). Dormancy does not simply represent arrested synaptic development because the dormant terminals identified after depolarization come from a previously active population (Moulder et al., 2004). One major outstanding unknown in the literature has been the pathway leading from depolarization to silent presynaptic terminals.

Prior studies have found that reduced cyclic adenosine monophosphate (cAMP)

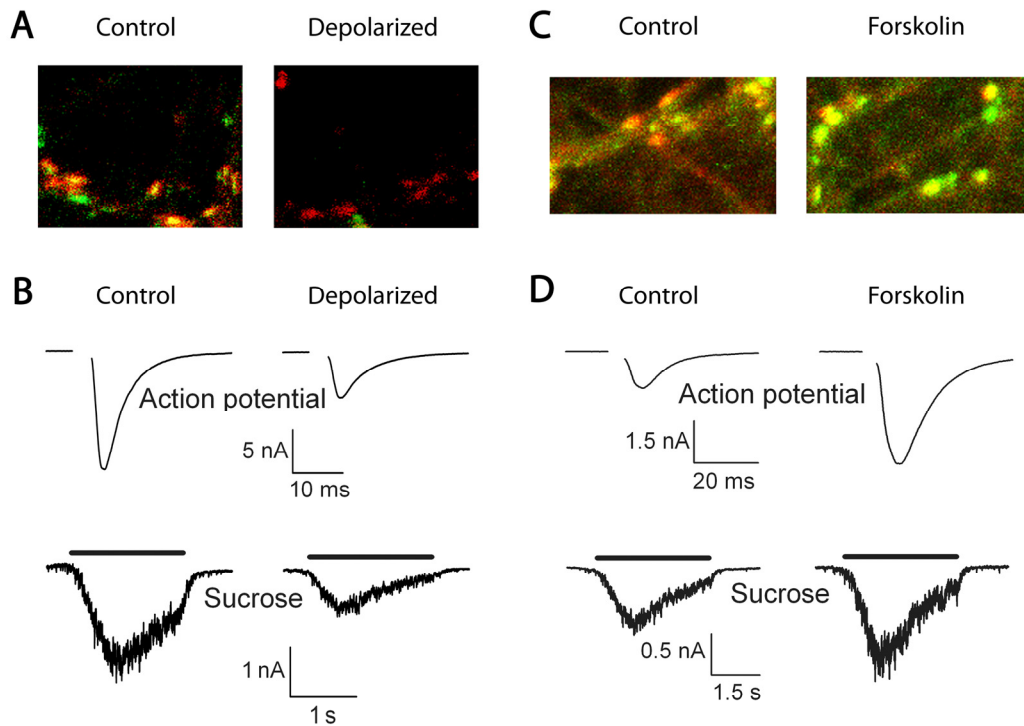


Figure 2. Malleability of the number of dormant presynaptic terminals. **A.** The percentage of active glutamate terminals is measured using FM1-43 dye uptake (green) and its co-localization with a presynaptic marker (vesicular glutamate transporter 1 or vGluT-1; red); dormant terminals are, therefore, represented by red puncta without any overlying green. Activity also modulates the number of dormant terminals. For example, co-localization occurs more often in control than in depolarized (4 h 30 mM KCl) cultured hippocampal neurons. This suggests that fewer glutamatergic presynaptic terminals are competent to recycle vesicles after depolarization. Modified with permission from Crawford et al., 2011 (Chapter 3). **B.** The percentage of active terminals correlates with the size of the readily releasable pool of vesicles. Excitatory postsynaptic currents (EPSCs) measured in autaptic cultured hippocampal neurons are depressed after prolonged (16 hr) depolarization with 30 mM KCl. Top: EPSCs were elicited after action potential stimulation, which are probabilistically dependent on calcium influx into the presynaptic terminal. Bottom: EPSCs were elicited by application of hypertonic sucrose, which causes calcium-independent fusion of all release-ready vesicles. Because both types of EPSCs are depressed after depolarization, this suggests that the size of the readily releasable pool of vesicles is decreased rather than the probability of vesicle release. **C.** The percentage of glutamatergic (red) terminals in cultured hippocampal neurons that take up the dye FM1-43 (green) increases after 4 h 50 μ M forskolin treatment, which increases adenylyl cyclase activity and, therefore, cAMP production. This suggests that more terminals are release-competent after increased cAMP signaling. Modified with permission from Moulder et al., 2008. **D.** EPSCs in autaptic hippocampal neurons are increased after 4 h 50 μ M forskolin application. Because both calcium-dependent action-potential evoked EPSCs and calcium-independent sucrose-evoked EPSCs are increased after forskolin, this suggests that the readily releasable pool is increased rather than the probability of vesicle release. Modified with permission from Moulder et al., 2008.

signaling increases the number of dormant presynaptic terminals (Moulder et al., 2008). This suggests that dormancy can be modulated through alterations in cAMP levels, potentially implicating adenylyl cyclase signaling as the upstream control point in dormancy induction. Because a major target of cAMP is protein kinase A (PKA), PKA substrates may modulate presynaptic vesicle release during dormancy induction (Fig. 3). One PKA substrate that is also involved in vesicle priming is the presynaptic protein Rim1 (Lonart et al., 2003; Calakos et al., 2004). Rim1 levels, and levels of an associated priming protein Munc13-1, are decreased in cultured hippocampal neurons after induction of depolarization-induced dormancy (Jiang et al., 2010). This is consistent with prior literature that suggests loss of Munc13-1 increases presynaptic muting (Augustin et al., 1999). Overexpression of Rim1 prevents depolarization from inducing presynaptic dormancy (Jiang et al., 2010), strengthening the evidence that the levels of priming proteins at the presynaptic terminal are vital for determining dormancy status. Although Rim1 and Munc13-1 are the only proteins found to be degraded during depolarization-induced muting at glutamatergic terminals (Jiang et al., 2010), genetic loss of other presynaptic proteins, like bassoon, also increases dormancy (Altrock et al., 2003). Interestingly, activity deprivation also reduces connectivity between CA3 pyramidal neurons in the hippocampus via presynaptic dormancy in a cyclin-dependent kinase 5 (CDK5)-dependent, but cAMP-independent, manner (Mitra et al., 2012). CDK5 may reduce connectivity between CA3 neurons by removing vesicles from the recycling pool (Kim and Ryan, 2010), although this remains unclear. Presynaptic proteins involved in vesicle coordination and release, therefore, appear to modulate dormancy through altered levels or function.

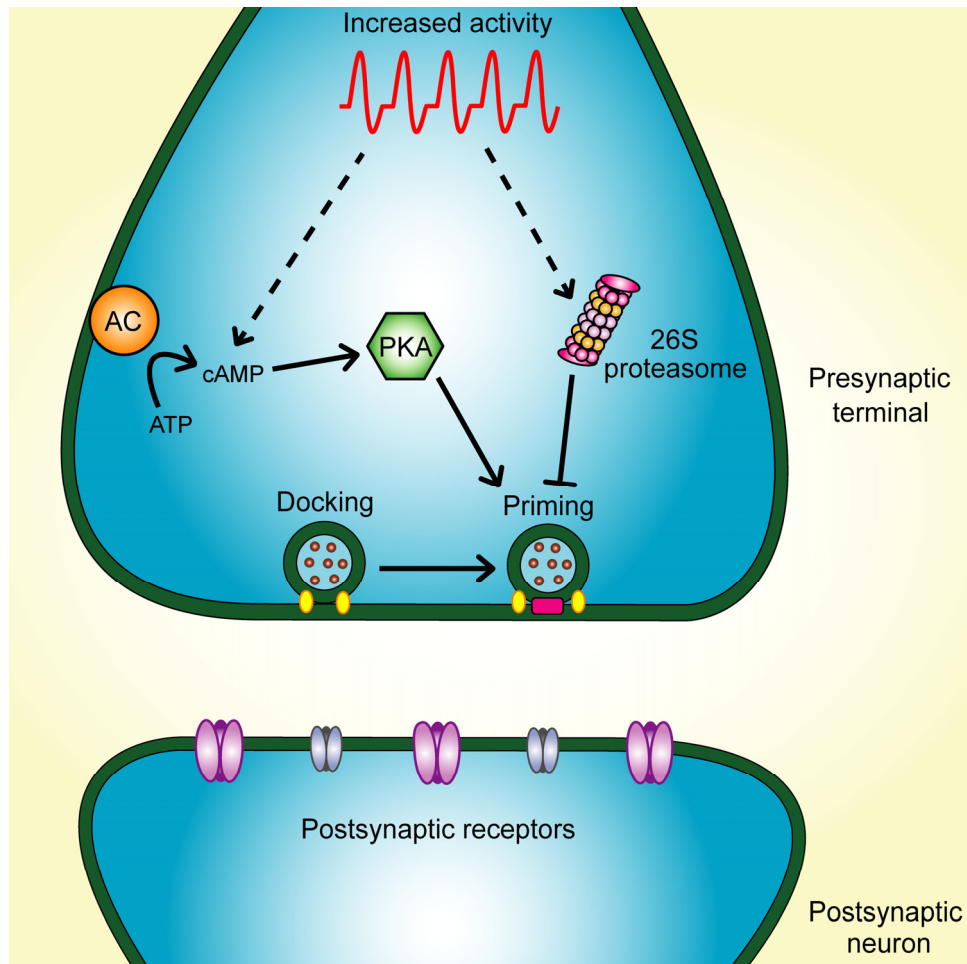


Figure 3. Signaling cascades participating in presynaptic dormancy induction. Prolonged strong depolarization or increased action potential firing induces presynaptic dormancy through reduction of cAMP signaling cascades and through activation of the ubiquitin-proteasome system. Depolarization increases proteasome activity through unknown mechanisms. Reduced cAMP likely reduces protein kinase A (PKA) signaling during silencing induction. PKA phosphorylates presynaptic priming proteins like Rim1, a modification that may render Rim1 resistant to proteasome degradation; therefore, less Rim1 phosphorylation is expected after depolarization. Increased proteasome activity, combined with a vulnerable presynaptic protein population, may then lead to priming protein degradation. This model provides a plausible mechanism for priming protein level reduction and dormancy induction by depolarization. Postsynaptic protein levels are unaltered by induction of presynaptic dormancy. Modified from Crawford and Mennerick, 2012.

The ubiquitin-proteasome system, a protein tagging and degradation pathway, is in a unique position to manipulate presynaptic dormancy. Increased degradation could decrease presynaptic protein levels and, therefore, cause dormancy in protein-deficient terminals. In fact depolarization- and hypoxia-induced silencing of presynaptic terminals are prevented by pharmacological proteasome inhibition (Jiang et al., 2010; Hogins et al., 2011). Together, these studies suggest that depolarization activates pathways to reduce cAMP and upregulate proteasome-dependent degradation of proteins vital for vesicle fusion at the presynaptic terminal (Fig. 3).

Induction of presynaptic unsilencing:

Depolarization increases dormancy, but neuronal inactivity reduces dormancy. Thus, activity levels appear to set the percentage of release-competent terminals. The percentage of active glutamate terminals in primary hippocampal cultures increases after 6-10 days of tetrodotoxin treatment to block action potentials (Moulder et al., 2006). Postsynaptic receptor blockade, but not activity deprivation, produces presynaptic unsilencing in less than 24 h (Jakawich et al., 2010). Unsilencing occurs within 4 h when dormancy-inducing depolarization is removed from neurons, which accounts for a return to baseline synaptic functionality (Moulder et al., 2004). Together, these studies suggest that presynaptic silencing is a compensatory, homeostatic adaptation to altered neuronal activity.

Signaling cascades for both silencing and unsilencing of presynaptic terminals appear to utilize cAMP. As described earlier, decreased cAMP signaling increases dormancy. In turn, increased cAMP signaling reduces dormancy (Fig. 2C, 2D, and 4).

Sp-cAMPS, a phosphodiesterase-resistant cAMP analog that activates downstream protein kinases like PKA, increases the number of functional release sites (Bolshakov et al., 1997; Ma et al., 1999; Kohara et al., 2001). This same effect is induced by forskolin, which increases adenylyl cyclase activity (Kohara et al., 2001; Moulder et al., 2008; Fig. 2C, D). Recovery from depolarization-induced silencing of glutamate terminals depends on adenylyl cyclase 8 and PKA signaling, suggesting that cAMP signaling is important for this type of unsilencing as well (Moulder et al., 2008). Interestingly, Sp-cAMPS-induced increases in the number of functional terminals are blocked with protein synthesis inhibitors (Ma et al., 1999), and the presynaptic protein synapsin has been implicated in a PKA-dependent form of presynaptic awakening (Cousin and Evans, 2011). A non-cAMP-dependent form of unsilencing requires postsynaptic brain-derived neurotrophic factor (BDNF) synthesis and release (Jakawich et al., 2010), although retrograde signaling of BDNF is contentious because BDNF is localized presynaptically (Dieni et al., 2012). The dependence of unsilencing on protein synthesis mirrors the evidence that protein degradation induces dormancy and strengthens the hypothesis that presynaptic protein levels or function are important for modulating dormancy status of presynaptic terminals.

Although dormancy is modulated homeostatically in some contexts, it may also be modulated in a Hebbian fashion. Some stimuli that induce long-term potentiation (LTP), a persistent enhancement of synaptic efficacy, also unsilence presynaptic terminals. Although the role of the presynaptic terminal in LTP induction is debated in the literature (Voronin and Cherubini, 2004; Kerchner and Nicoll, 2008; Kullmann, 2012), there are a few studies that show an increase in the number of active presynaptic

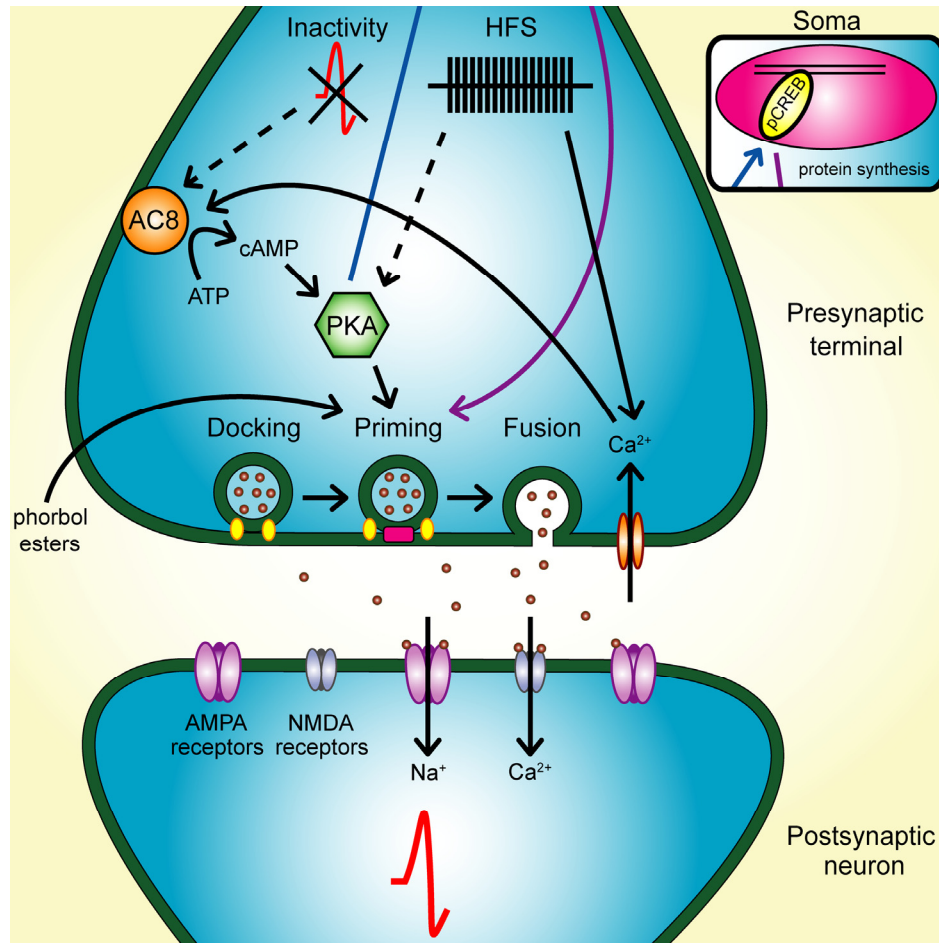


Figure 4. Signaling cascades participating in presynaptic dormancy reduction. Under some conditions, inactivity and increased cAMP activate previously dormant terminals. Calcium-dependent adenylyl cyclase VIII (AC8) knockout prevents recovery of active terminals when elevated neuronal activity is removed, so AC8 may be the cAMP source responsible for this form of unsilencing. In other contexts multiple bursts of high frequency stimulation (HFS), which often causes strong calcium influx and long-term potentiation, lead to presynaptic awakening. Both activity reduction and HFS require protein kinase A (PKA) signaling for presynaptic activation, but how ostensibly opposite changes in activity both recruit PKA in different experimental contexts remains unknown. Once PKA is activated, phosphorylation events may slow priming protein degradation. Additionally, PKA phosphorylates and thereby activates nuclear transcription factors (blue arrow and inset) like cAMP response element-binding protein (CREB), and this may increase synthesis of presynaptic proteins vital for vesicle priming and release (purple arrow and inset). A third pathway to unsilencing is via phorbol esters, which enhance function of the priming protein Munc13-1. Under this model of dormancy reduction, postsynaptic responses are restored once the presynaptic terminal regains the ability to release neurotransmitter.

terminals after LTP has been induced. In one study, for example, Sp-cAMPS, known to reduce dormancy, potentiates synapses and increases quantal n in rodent hippocampal slices (Bolshakov et al., 1997). Another study used multiple bursts of high frequency stimulation in cultured dentate granule neurons to induce a form of PKA-dependent LTP. Using a slowly reversible NMDA receptor antagonist, MK801, to block postsynaptic receptors at all active synapses, this study showed that new active release sites appear after LTP induction (Tong et al., 1996). PKA is also important for activation of presynaptic terminals in immature cultured hippocampal neurons after repetitive depolarization challenges, although the possibility of enhanced synaptic maturation rather than awakening of established terminals is not ruled out (Yao et al., 2006). Another study in more mature cultured hippocampal neurons showed that glutamate-induced synaptic potentiation increases the number of terminals that stain for FM1-43 (Ninan and Arancio, 2004). Hebbian unsilencing of presynaptic terminals is also induced by serotonin in *Aplysia* sensory neurons prior to subsequent synaptogenesis (Kim et al., 2003). Presynaptic dormancy modulation, therefore, may be important for some Hebbian forms of synaptic plasticity, and the direction of cAMP change may critically determine whether silencing or unsilencing results from a particular stimulus.

Phorbol esters, which are analogues of diacylglycerol, also potentiate synaptic function. Among other presynaptic effects, phorbol esters unsilence presynaptic terminals within two minutes application to cultured hippocampal neurons (Chang et al., 2010). This form of unsilencing is much faster than the cAMP-dependent forms of unsilencing described previously. Phorbol esters upregulate protein kinase C signaling, but they also act directly on Munc13-1 (Betz et al., 1998). Although PKA-induced

unsilencing may involve protein synthesis, phorbol ester-induced unsilencing is likely too rapid to require protein synthesis. Instead, phorbol esters appear to alter Munc13-1 function, potentially via translocation to the plasma membrane (Betz et al., 1998). Altogether, these findings suggest that there are multiple signaling cascades that cause presynaptic unsilencing (Fig. 4). These cascades alter synaptic function on very different time scales, but they likely converge on presynaptic proteins responsible for vesicle maturation or fusion.

Implications of presynaptic dormancy:

Because dormant presynaptic terminals are widespread and modulated by a variety of mechanisms, dormancy status may represent an important facet of synaptic malleability. It is currently unknown how dormancy levels affect network function and what role this may play during neuropathology. Why should some synapses exist but not function? What benefit or difference in function is gained from dormancy over a more classical presynaptic change, like acute, reversible G-protein-coupled receptor (GPCR)-mediated depression of calcium influx and the corresponding decreased vesicle release probability (Brown and Sihra, 2008)? An intriguing possibility is that dormancy is an activity-dependent mechanism for modulating connectivity between neurons. This would add another dimension to neural computation since dormancy provides a digital signal that is either “on” or “off,” similar to synaptogenesis and synaptic pruning but without the same resource requirements of these mechanisms. Having multiple methods for modulating synaptic connectivity likely increases flexibility of neural computation in the system.

It is also interesting that dormancy can be adaptive or reinforcing, depending on the context and the direction of cAMP change. Presynaptic muting of glutamate terminals often increases with prolonged elevated neuronal activity but decreases with inactivity. This counteractive change in excitatory neurotransmitter release could potentially maintain signaling within an optimal range, one of the hypothesized roles of homeostatic synaptic plasticity (Pozo and Goda, 2010). For example, induction of dormancy occurs during pathological insults, leading to reduced glutamate release and enhanced neuronal survival (Hogins et al., 2011; Appendix). In this context, all-or-none muting seems well-tailored to self-defense; other forms of presynaptic depression through reduced calcium influx might quickly be overwhelmed by hypoxic or ischemic depolarizing insults. Dormancy can also be decreased rather than increased after stimulation, however, leading to potentiated synapses. Some of these Hebbian forms of unsilencing utilize cAMP signaling, which, like LTP, has been linked to memory processing in multiple systems (Alberini et al., 1995). So in addition to its potentially homeostatic role, modulation of dormancy may also play a role in Hebbian plasticity and memory. Therapeutic exploitation of the signaling cascades increasing or decreasing dormancy could have valuable clinical implications.

Dormancy may also be more efficient than ostensibly similar forms of synaptic plasticity. Modulation of dormancy achieves similar changes in neuronal connectivity as synaptogenesis and synaptic pruning but appears to work on a faster time scale (minutes to hours rather than hours to days). This likely preserves physical resources the cell would otherwise require for large-scale structural alterations and allows the neuron to respond more quickly to perturbations to the system. Additionally, it is important to note

that presynaptic function requires a large amount of energy, as evidenced by the nearly ubiquitous presence of mitochondria in presynaptic terminals (Palay, 1956). If the system requires a dormant synaptic connection, it would arguably waste less energy to preserve postsynaptic function and shut down presynaptic vesicle cycling than to preserve transmitter release onto a non-receptive postsynaptic membrane for the duration of the dormancy period. Although it is unclear whether unsilencing a presynaptic terminal would require more energy than unsilencing a postsynaptic terminal, maintaining presynaptically silent synapses may be more energy efficient than maintaining postsynaptically silent synapses. Therefore, it is unclear how presynaptically silent and postsynaptically silent synapses interact. It is also unclear how the relatively fast digital changes in information flow created by dormancy alter neural computation, but it is evident that presynaptically silent terminals are a potentially economical way to introduce this dimension into the system. Clearly, understanding the mechanisms responsible for muting induction and its implications would be of benefit.

Mediators of synaptic plasticity

Calcium:

Calcium is a well-studied participant in neuronal and synaptic plasticity induction, but it is unknown if presynaptic muting induction requires calcium signaling. Calcium influx into the presynaptic terminal is vital for action potential-evoked synaptic transmission (Sudhof, 2004, 2012), but calcium can also modulate other signaling cascades within the neuron, including cAMP levels (Hanoune and Defer, 2001). Induction of many persistent, Hebbian forms of synaptic plasticity requires calcium signaling (Malenka, 1991; Malenka, 1994; Teyler et al., 1994). For example, NMDA receptor-dependent LTP of synapses onto CA1 pyramidal neurons in the hippocampus require postsynaptic calcium influx (Wigstrom et al., 1979; Lynch et al., 1983; Malenka et al., 1988; Malenka et al., 1992). LTP of inhibitory synapses onto CA1 pyramidal cells after theta burst electrical stimulation and LTP of mossy fiber synapses onto CA3 pyramidal cells are likewise dependent on postsynaptic calcium (Williams and Johnston, 1989; Patenaude et al., 2003). Both LTP and its Hebbian counterpart, long-term depression (LTD), onto dentate granule cells in the hippocampus are inhibited by postsynaptic calcium chelation (Xie et al., 1992). LTD onto CA1 pyramidal neurons as well as mossy fiber LTD onto CA3 neurons also require calcium (Wickens and Abraham, 1991; Mulkey and Malenka, 1992; Tzounopoulos et al., 1998). Calcium is vital, therefore, for many Hebbian forms of plasticity.

Calcium dependence of Hebbian plasticity is not just observed in intact hippocampal networks, however. LTP induced in cultured dentate granule neurons and

the awakening of presynaptic terminals in dissociated hippocampal neurons after high-frequency stimulation both require presynaptic calcium (Tong et al., 1996; Shen et al., 2006). Autaptic hippocampal neurons grown in culture require the release of calcium from intracellular calcium stores for maintenance of CB1 cannabinoid receptor-dependent LTD (Kellogg et al., 2009). These culture systems recapitulate basic findings found in more intact networks.

Despite the large number of Hebbian forms of plasticity that require calcium, some do not. For example, some hippocampal forms of LTD that depend on metabotropic glutamate receptors (mGluRs) are induced despite calcium chelation (Fitzjohn et al., 2001; Ireland and Abraham, 2009; Kasten et al., 2012). Similarly, unsilencing of cerebellular granule presynaptic terminals via cAMP signaling does not require calcium (Chavis et al., 1998). Depotentiation of Schaffer collateral LTP by temporoammonic path stimulation does not require L-type calcium channels, although it is unclear if calcium from other sources contributes (Izumi and Zorumski, 2008). Calcium is, therefore, a common but not ubiquitous signal mediating many forms of Hebbian plasticity.

The contributions of calcium signaling to other forms of neuronal and synaptic plasticity have also been studied in detail. Subtypes of homeostatic and short-term plasticity have been found to require calcium (Fisher et al., 1997; Fioravante and Regehr, 2011; Mochida, 2011; Turrigiano, 2012). For example, activity in CA1 pyramidal neurons causes calcium influx through L-type channels that subsequently increases spike adaptation via potassium channels (Wu et al., 2008). Although this may be a homeostatic mechanism for reducing excitability, some calcium-dependent changes in intrinsic

excitability are reinforcing, like the increased excitability in layer V cortical neurons after electrical stimulation (Cudmore and Turrigiano, 2004). In *Drosophila*, postsynaptic glutamate receptor blockade increases presynaptic glutamate release via increased vesicle release probability. This homeostatic effect is blocked in mutants with reduced functional Cav2.1, a presynaptic voltage-gated calcium channel (Frank et al., 2006). In cultured rat cortical neurons, activity blockade homeostatically increases postsynaptic AMPA receptor levels in a process known as synaptic scaling. The AMPA receptor increase is triggered by decreased calcium influx into the postsynaptic neuron (Ibata et al., 2008). Although short-term depression of synaptic transmission via vesicle depletion does not require calcium (Betz, 1970; Stevens and Tsujimoto, 1995; Garcia-Perez et al., 2008), recovery from this depression does (Garcia-Perez and Wesseling, 2008). Other forms of synaptic plasticity that do not require calcium include nitric oxide-induced neurotransmitter release via vesicle-associated protein assembly as well as hormone-induced increases in AMPA receptor levels (Meffert et al., 1996; Zadran et al., 2009). Calcium is an important messenger for many forms of plasticity, but it remains unknown if calcium mediates presynaptic muting induction.

G-proteins:

Another group of molecular signals important for many forms of synaptic plasticity is that of guanine nucleotide-binding proteins (G-proteins). G-proteins are heterotrimeric protein complexes associated with GPCRs, which are transmembrane receptors that alter their conformation upon ligand binding. Activated receptors convert guanosine diphosphate bound to the $G\alpha$ subunit to guanosine triphosphate. Canonically,

this causes dissociation of the $G\alpha$ subunit and the $G\beta\gamma$ subunit complex from the receptor, allowing these subunits to bind to and alter the activity of downstream effectors. In the nervous system, G-proteins mediate multiple functional and modulatory roles, including synaptic plasticity (Brown and Sihra, 2008). G-protein signaling cascades, therefore, are well-poised to modulate neural communication, but it is unknown if they play a role during glutamatergic synaptic maturing.

Many different signaling cascades lie downstream of G-protein activation, but one important downstream target is cAMP. The $G\alpha_s$ subunit stimulates cAMP production through activation of adenylyl cyclase, an enzyme that catalyzes the formation of cAMP from adenosine triphosphate (ATP). In contrast, $G\alpha_i$ and $G\alpha_o$, which are sensitive to pertussis toxin, inhibit adenylyl cyclase. In neurons, acute activation of inhibitory G-proteins ($G_{i/o}$) also induces rapidly reversible effects on ion channels through $G\beta\gamma$ signaling. $G\beta\gamma$ causes synaptic depression through presynaptic inhibition of calcium channels that reduces vesicle p_r . $G\beta\gamma$ signaling also decreases neuronal excitability through postsynaptic activation of inwardly rectifying potassium channels that hyperpolarizes the neuron (Brown and Sihra, 2008). These acute effects of $G\beta\gamma$ on ion channels do not desensitize quickly, as evidenced by their ability to be elicited after 4 h exposure to GPCR agonist (Wetherington and Lambert, 2002a, b), suggesting that this is a robust mechanism for altering neuronal function. G-protein signaling cascades are diverse but utilize common mechanisms to alter intracellular processes.

G-protein signaling is involved in more slowly induced synaptic changes as well. One well-studied example is LTD of excitatory synapses onto CA1 pyramidal neurons in the hippocampus. This form of LTD is induced by stimulation of mGluRs, which are one

type of GPCR expressed in the hippocampus (Oliet et al., 1997; Palmer et al., 1997; Kasten et al., 2012). Similarly, mossy fiber synapses onto CA3 pyramidal neurons in the hippocampus produce an NMDA receptor-independent, mGluR-dependent LTD that requires decreases in cAMP levels (Tzounopoulos et al., 1998). Interestingly, mGluR-independent depotentiation of Schaffer collateral LTP by temporoammonic path stimulation requires A1 adenosine receptor activation (Izumi and Zorumski, 2008), a GPCR in excitatory presynaptic terminals and astrocytes (Yoon and Rothman, 1991; Biber et al., 1997). Although LTD of inhibitory synapses onto CA1 pyramidal neurons can be induced by mGluR stimulation (Patenaude et al., 2003), CB1 cannabinoid receptors, another class of hippocampal GPCR, also mediate a form of inhibitory synapse LTD in this region (Chevalleyre and Castillo, 2004). Activation of CB1 cannabinoid receptors also produces presynaptic muting of GABAergic terminals in hippocampal slices (Losonczy et al., 2004), but it is unclear how this interacts with LTD. It is also unknown if G-protein signaling is involved in other forms of muting like in hippocampal glutamatergic synapses.

Classical G-protein signaling requires extracellular ligand binding to GPCRs, so intercellular communication mediates most G-protein signals. Cell autonomous mechanisms for activating GPCRs exist, however. Some GPCRs or downstream targets are voltage-sensitive (Reddy et al., 1995; Ben-Chaim et al., 2006; Parnas and Parnas, 2007; Okamura et al., 2009), so neuronal depolarization or increased action potential firing could modulate G-protein signaling through voltage signals. Additionally, activators of G-protein signaling (AGS proteins) are recently-described modulators of G-protein subunits that do not require GPCR activation (Cismowski, 2006; Blumer et al.,

2007; Blumer et al., 2012). AGS proteins are expressed in the brain, including the hippocampus, but their role in neuronal function and their activation mechanisms are still being investigated (Fang et al., 2000; Takahashi et al., 2003; Schwendt and McGinty, 2010). These examples highlight the diversity in G-protein signaling cascades that alter neuronal and synaptic function.

Astrocyte-derived molecular signals:

Glial cells have historically been regarded as support cells for the neurons that relay information in the nervous system. The name “glia” was originally derived from a Greek word for “glue” to describe their role in physically supporting neural networks. Glial promotion of blood-brain barrier integrity, neuronal development, electrical signal propagation, immune function, and transporting waste from the extracellular space have been well-studied (Abbott et al., 2010; Sofroniew and Vinters, 2010; Saijo and Glass, 2011; Nualart-Marti et al., 2012; Parpura et al., 2012). One important contribution that astrocytes, one glial type, make to neuronal development is promotion of synaptogenesis (Eroglu and Barres, 2010). Factors released by astrocytes increase the total number of synapses and speed synaptic development (Hama et al., 2004; Christopherson et al., 2005; Eroglu et al., 2009; Xu et al., 2010; Kucukdereli et al., 2011). Astrocytes and other glial cells are important for the development and function of neurons.

Recently, it was discovered that astrocytes communicate with neurons to modulate neuronal and synaptic function more acutely than previously thought. Astrocytes release neurotransmitters, neuromodulators, and precursors to neuromodulators via transporters or vesicle fusion to modulate neural function (Volterra

and Meldolesi, 2005; Bergersen and Gundersen, 2009; Hamilton and Attwell, 2010; Paixao and Klein, 2010; Debanne and Rama, 2011; Santello et al., 2011; Min and Nevian, 2012). Examples of these “gliotransmitters” include the neurotransmitter glutamate (Parpura et al., 1994; Bonansco et al., 2011), the NMDA receptor co-agonist D-serine (Mothet et al., 2005; Oliet and Mothet, 2006; Debanne and Rama, 2011), and ATP that is converted to adenosine in the extracellular space (Queiroz et al., 1997; Pascual et al., 2005; Butt, 2011). This newly described ability of glial cells is controversial and its physiological relevance remains to be determined.

Many reports have described effects of astrocytes on synaptic plasticity. Although astrocytes are thought to mediate many forms of synaptic function via downstream events after intracellular calcium rises (Volterra and Meldolesi, 2005; Perea et al., 2009; Dityatev et al., 2010; Halassa and Haydon, 2010; Di Castro et al., 2011; Dityatev and Rusakov, 2011; Honsek et al., 2012), this property is not universal and is sometimes contentious (Fiacco et al., 2007; Agulhon et al., 2010; Halassa and Haydon). In the hippocampus, calcium-dependent D-serine release from astrocytes is required for LTP of Schaffer collateral synapses onto CA1 pyramidal neurons (Henneberger et al., 2010). Additionally, astrocyte-derived tumor necrosis factor α is required, but not directly instructive, in hippocampal and cortical neurons for homeostatic scaling of glutamate receptor levels after activity deprivation (Beattie et al., 2002; Stellwagen and Malenka, 2006; Perea et al., 2009; Steinmetz and Turrigiano, 2010; Sullivan et al., 2011). SPARC (secreted protein, acidic, rich in cysteine) is a factor released by astrocytes that promotes AMPA receptor stabilization and prevents their over-accumulation and may, therefore, modulate synaptic strength (Jones et al., 2011; Kucukdereli et al., 2011).

Interestingly, microglia increase spontaneous activity onto CA1 pyramidal neurons through activation of astrocytic purinergic receptors upstream of neuronal mGluR activation (Pascual et al., 2011). Outside of the hippocampus, glial cells have been linked to synaptic plasticity or acute changes in neuronal function in rodent barrel cortex (Eroglu et al., 2009; Benedetti et al., 2011; Takata et al., 2011), hypothalamus (Gordon et al., 2009), thalamus (Pirttimaki et al., 2011), cerebellum (Lee et al., 2010), septal nucleus (Navarrete et al., 2012), trigeminal ganglion (Ceruti et al., 2011), and neuromuscular junction (Todd et al., 2010). One study, however, found that controlled calcium elevations in astrocytes did not increase spontaneous EPSC frequency or amplitude onto CA1 pyramidal neurons and had no effect on LTP (Agulhon et al., 2010). This study questions the role of astrocytic calcium transients in synaptic plasticity but does not rule out the possibility that astrocytes communicate through other means to modulate synapses. Additionally, the source of adenosine involved in synaptic depression after excitatory synapse stimulation was found to be neuronal and not astrocytic (Lovatt et al., 2012), as had been hypothesized. Despite the evidence that astrocytes play an important role in synapse development and plasticity in some contexts, it remains unclear whether astrocytes modulate presynaptic muting induction.

Summary and thesis objectives

Clarifying the induction signals responsible for presynaptic muting will increase our understanding of synaptic modulation and also potentially lead to new therapeutics for nervous system insults. For this reason, the overall aim for this dissertation project is to elucidate the molecular signals responsible for muting induction. Downstream expression mechanisms are better understood than upstream induction signals, so I will focus on upstream triggers for muting. I will study muting in response to depolarization because it is a robust phenomenon with clear neuroprotective effects during excitotoxic injury.

Cultured hippocampal neurons are used as the model system for studying muting induction for many reasons. Not only is the hippocampus a vital structure for learning and memory (Naber et al., 2000; Silva et al., 2009), but it is also well-known for long-term synaptic plasticity and is vulnerable to excitotoxic injury (Cervos-Navarro and Diemer, 1991; Davolio and Greenamyre, 1995; Harry and Lefebvre d'Hellencourt, 2003; Kemp and Manahan-Vaughan, 2007; De Roo et al., 2008; Howland and Wang, 2008). Studying hippocampal synaptic plasticity, therefore, could have broad implications in human health and disease. Additionally, early work from our lab found muting of glutamatergic hippocampal synapses to be robust, reproducible, and induced with relatively brief challenges that do not cause other, confounding synaptic changes (Moulder et al., 2004; Moulder et al., 2006). Small networks of neurons also allow for clear separation and interpretation of electrical and optical signals gathered from electrophysiology and microscopy, respectively. Importantly, manipulation of molecular signals is more simply and completely achieved in a culture system where cell layers,

density, and identity are controlled. Dissociated hippocampal cultures, therefore, are a useful model system for this project.

A major underlying question for the work described herein is whether muting induction is cell autonomous or whether it requires intercellular signaling. If muting induction is cell autonomous, for example via calcium influx during neuronal depolarization, then neurons likely sense increased activity and subsequently decrease their output of neurotransmitter without further input from the environment. For this reason, I will attempt to develop methods for depolarizing individual neurons for long periods of time through heterologous cation channel expression. The involvement of non-cell autonomous mechanisms, like ligand-gated GPCR activation or astrocytic modulation of muting, would imply that the neural network contributes to this homeostatic process. Calcium, G-proteins, and astrocyte signaling will be tested because of their established roles in synaptic plasticity and potential to interact with known mediators of muting. Clarifying mechanisms of muting induction could answer fundamental questions about how the nervous system adapts to changes in network activity levels.

In summary, synapses represent the main junctures of communication between neurons in the nervous system. In many neurotransmitter systems, a fraction of presynaptic terminals fails to release vesicles in response to action potential stimulation and strong calcium influx. These silent presynaptic terminals exhibit a reversible functional dormancy beyond low vesicle release probability, and dormancy status may have important implications in neural function. Recent advances have implicated presynaptic proteins interacting with vesicles downstream of cAMP and protein kinase A

signaling cascades in modulating the number of these mute presynaptic terminals, but it remains unclear how these changes are induced. Muting may represent a homeostatic neuroprotective mechanism active during pathological insults involving excitotoxicity, but it may also be induced during Hebbian plasticity. The goals of this dissertation are to ask whether important mediators of other forms of synaptic plasticity, like calcium, G-proteins, and factors released by astrocytes, also mediate muting induction. This project aims to clarify molecular signaling cascades responsible for muting induction and identify potential targets for therapeutic intervention.

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Chapter 2

Comparative effects of heterologous TRPV1 and TRPM8 expression in rat hippocampal neurons

This chapter contains a previously published manuscript:

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Author contributions for the citation above:

D.C.C. designed and performed experiments, analyzed data, and wrote the paper. K.M. designed and performed experiments. R.W.G. provided reagents and designed experiments. G.M.S. provided reagents. S.M. designed and analyzed experiments and wrote the paper.

Abstract

Heterologous channel expression can be used to control activity in select neuronal populations, thus expanding the tools available to modern neuroscience. However, the secondary effects of exogenous channel expression are often left unexplored. We expressed two transient receptor potential (TRP) channel family members, TRPV1 and TRPM8, in cultured hippocampal neurons. We compared functional expression levels and secondary effects of channel expression and activation on neuronal survival and signaling. We found that activation of both channels with appropriate agonist caused large depolarizing currents in voltage-clamped hippocampal neurons, exceeding the amplitude responses to a calibrating 30 mM KCl stimulation. Both TRPV1 and TRPM8 currents were reduced but not eliminated by 4 hr incubation in saturating agonist concentration. In the case of TRPV1, but not TRPM8, prolonged agonist exposure caused strong calcium-dependent toxicity. In addition, TRPV1 expression depressed synaptic transmission dramatically without overt signs of toxicity, possibly due to low-level TRPV1 activation in the absence of exogenous agonist application. Despite evidence of expression at presynaptic sites, in addition to somatodendritic sites, TRPM8 expression alone exhibited no effects on synaptic transmission. Therefore, by a number of criteria, TRPM8 proved the superior choice for control over neuronal membrane potential. This study also highlights the need to explore potential secondary effects of long-term expression and activation of heterologously introduced channels.

Introduction

There has been strong recent interest in heterologous control over electrical activity in neurons and other excitable cells (Miesenbock, 2004; Deisseroth et al., 2006; Gorostiza and Isacoff, 2008). These approaches offer the possibility of remotely controlling the activity of select populations of neurons, thereby gaining experimental or therapeutic influence over network activity. For instance, heterologously introduced channels have been used recently to control courtship and escape behavior in flies and fish (Clyne and Miesenbock, 2008; Douglass et al., 2008; Peabody et al., 2009; Zimmermann et al., 2009) and to control sleep-wake behavior and motor behavior, including Parkinsonian and epileptic symptoms, in mammals (Adamantidis et al., 2007; Aravanis et al., 2007; Arenkiel et al., 2007; Arenkiel et al., 2008; Gradinaru et al., 2009; Tonnesen et al., 2009). Because electrical activity is critical for neuronal survival, development, and plasticity (Zito and Svoboda, 2002; Akerman and Cline, 2007; Li et al., 2009), these techniques also have experimental potential to help unravel the downstream signaling mechanisms responsible for these important facets of neuronal function (Burrone et al., 2002; Sohal et al., 2009).

Heterologous expression of ligand-gated receptors and leak channels received initial attention (Burrone et al., 2002; Zemelman et al., 2003; Lima and Miesenbock, 2005; Hou et al., 2008; Okada and Matsuda, 2008), but this approach has largely been supplanted by optogenetic approaches that employ channels directly activated by light (Chambers et al., 2006; Zhang et al., 2006; Szobota et al., 2007; Han et al., 2009). Multiple approaches for heterologous control over activity are probably needed, however, as one single method is unlikely to be appropriate in all situations. Introduction of leak

channels (Burrone et al., 2002; Hou et al., 2008; Okada and Matsuda, 2008) suffers from a lack of control over the degree of activity change and typically is limited to an inhibitory influence, and one obvious disadvantage of optogenetic approaches is the need for a light source. A light source of appropriate wavelength will not be feasible or cost-effective in all circumstances. In the intact nervous system, light implantation may be prohibitively invasive. Furthermore, some applications may require activation of a spatially dispersed set of neurons over a large area, in which case a single light source may not be adequate.

For these reasons, we have explored further the advantages and disadvantages of a heterologous ligand-gated receptor approach. This approach offers the possibility of global administration of ligand for activating populations of heterologously transfected neurons. We explored two candidate heterologous ligand-gated channels: transient receptor potential vanilloid receptor 1 (TRPV1) and transient receptor potential cation channel, subfamily M, member 8 (TRPM8), which normally are responsible for pain and temperature signal transduction in sensory neurons and respond to the pharmacological ligands capsaicin and menthol, respectively (Caterina et al., 1997; McKemy et al., 2002; Peier et al., 2002). These channels were chosen for their ability to be controlled readily by agonist application and their lack of strong endogenous role in hippocampal function. To test advantages and disadvantages of expressing each channel in hippocampal neurons, we used sparse transfection of neurons to avoid complications and confounds of widespread neuronal activation and associated release of neurotransmitters and modulators. We tested the activation of these channels relative to depolarizing currents activated by elevated extracellular potassium as a calibration standard.

Acute activation of both TRPV1 and TRPM8 induced large currents in response to saturating agonist concentration. Both showed evidence of desensitization with prolonged receptor activation, although these responses still compared favorably with non-desensitizing potassium-induced depolarizing currents. TRPM8 exhibited less agonist-induced toxicity and less interference with normal synaptic communication in the absence of agonist. In addition to soma expression of channels, we observed evidence for direct depolarization of axon terminals of TRPM8- and TRPV1-transfected neurons, suggesting cell-wide expression. Because our results suggest that TRPM8 is expressed throughout the cell with no detectable secondary effects on cell function, TRPM8 is the better choice for studies requiring exogenous control over the membrane potential of neuronal subpopulations. Our results also stress the importance of fully characterizing a heterologous expression system to avoid confounding and other unintended effects of manipulating that system.

Materials and Methods

Tissue culture and transfection:

All experimental procedures involving animals were performed using protocols approved by the Washington University in St. Louis School of Medicine Animal Studies Committee as well as the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health. Primary hippocampal neuron cultures were prepared as previously described (Mennerick *et al.*, 1995). Briefly, postnatal day 0-3 rat pups were anesthetized with isoflurane and decapitated. Hippocampi were removed, cut into 500 μM -thick transverse slices, and treated enzymatically with 1 mg/mL papain. Cells were then mechanically dissociated and plated as mass cultures (~ 650 cells/ mm^2 onto a uniform layer of collagen) or as microcultures (~ 100 cells/ mm^2 onto “islands” made of collagen droplets). The plating medium used was Eagle’s medium (Invitrogen, Carlsbad, CA, USA) supplemented with heat-inactivated horse serum (5%), fetal bovine serum (5%), 17 mM D-glucose, 400 μM glutamine, 50 U/mL penicillin, and 50 $\mu\text{g}/\text{mL}$ streptomycin. Cultures were housed in a humidified incubator at 37°C under controlled atmospheric conditions (5% CO_2 / 95% air). To inhibit cell division 3-4 days after plating, 6.7 μM cytosine arabinoside was added. Half of the culture medium was replaced with Neurobasal medium (Invitrogen) with B27 supplement 4-5 days after plating. Transfections were performed 8-12 days after plating using a DNA:Lipofectamine 2000 (Invitrogen) ratio of 1:1.5-2 according to the manufacturer’s protocol. Dishes were transfected with synaptophysin-YFP (0.5-2.5 μg), TRPV1 (2 μg), and/or TRPM8 (1.5 μg). Experiments were performed usually 1 day, but up to 3 days,

after transfection.

Electrophysiology:

Whole-cell voltage-clamp and cell-attached patch experiments were performed using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA), Digidata 1322A acquisition board (Molecular Devices), and pClamp software (Molecular Devices). All recordings were made in voltage-clamp mode at room temperature (~25°C). Whenever solutions were acutely applied to neurons, a multi-barrel perfusion system was used with the perfusion port placed < 0.5 mm from the neuron under study. For all experiments, controls consisted of either neurons from sibling cultures plated the same day or non-transfected neurons within the same dish as the transfected neurons under study unless otherwise described. All experiments, except for those depicted in Figure 6, were performed in conventional mass cultures.

Membrane potential was held at -70 mV for experiments unless otherwise indicated. Electrode resistances of 6-11 M Ω were used for cell-attached patch recordings. For all other recordings, electrode resistances were 2.5-6 M Ω . A 5 kHz low-pass filter was used for all experiments except cell-attached patches in which 1kHz filtering was utilized. Access resistance was compensated 85-100% for all experiments except where small currents were measured: cell-attached patches, miniature postsynaptic current recordings, acute agonist applications to non-transfected neurons, and acute ruthenium red applications to transfected and non-transfected neurons. Action potential stimulation for the autaptic recordings was achieved by 1.5 ms voltage pulses from -70 mV to 0 mV (Mennerick et al., 1995).

Extracellular recording media consisted of 138 mM NaCl, 4 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, 10 mM HEPES, and 25 μM D-APV (Tocris, Bristol, UK) with a pH of 7.25. For all agonist-induced current recordings (i.e. excluding microisland autaptic current and miniature postsynaptic current recordings), 1 μM NBQX (Tocris) was added to the extracellular media to inhibit secondary glutamate-mediated synaptic currents. For most experiments, internal pipette solution consisted of 130 mM cesium methanesulfonate (CsMeSO₄), 4 mM NaCl, 0.5 mM CaCl₂, 5 mM EGTA, and 10 mM HEPES at a pH of 7.25. For cell-attached patch experiments, however, 140 mM K-gluconate was substituted for CsMeSO₄, and for microisland autaptic recordings, 140 mM KCl was substituted for CsMeSO₄ to provide similar driving force on excitatory and inhibitory postsynaptic currents (PSCs) at the holding potential of -70 mV.

For some experiments, agonist-induced responses were compared with responses elicited by 30 mM KCl application. Prolonged exposure (4 hr) to 30 mM KCl has been shown to induce strong synaptic change in cultured hippocampal neurons and clamps the membrane potential near -20 mV (Moulder et al., 2004; Moulder et al., 2006). Normalizing the agonist-induced current to the KCl current, therefore, controls for variability in absolute current size due to neuronal geometry and provides a within-cell comparator to a calibrated depolarizing stimulus.

Epifluorescence imaging and cell counts:

All imaging was performed using an Eclipse TE2000-S inverted microscope (Nikon, Melville, NY, USA) with epifluorescence provided by a metal halide lamp. Light was routed through Chroma filter set 41001 (Chroma Technology Corp.,

Rockingham, VT, USA), with an HQ480/40 nm excitation filter and an HQ535/50 nm emission filter, and through Chroma filter set 41002 (Chroma Technology Corp.), with an HQ535/50 nm excitation filter and an HQ610/75 nm emission filter. Images were acquired using Metamorph software (Universal Imaging, Downingtown, PA, USA) with a cooled 12-bit CCD camera (Photometrics, Tucson, AZ, USA) through a 40X objective (0.6 numerical aperture). To determine transfection efficiency, the number of transfected and non-transfected neurons was counted in 10 randomly-selected fields for each culture dish by an observer naïve to the DNA combination. For cell health experiments, dishes were fixed for 10 min in 4% paraformaldehyde / 0.2% glutaraldehyde after agonist treatments. After washing 3 times in phosphate-buffered saline, transfected neurons were assessed for health under 40X magnification by an observer naïve to both the treatment conditions and the DNA combination.

Data analysis:

For all electrophysiological experiments, data were analyzed using Clampfit 9.2 (Molecular Devices) and Excel (Microsoft, Redmond, WA, USA) software. Graphs and traces were plotted using SigmaPlot software (SPSS Science, Chicago, IL, USA). Transfection efficiency was calculated by averaging the percentage of transfected neurons in each dish. This percentage was determined by dividing the number of transfected neurons by the total number of neurons counted in 10 fields and multiplying by 100. For Figures 1 and 3, an action potential was counted if there was a 1-10 pA transient and primarily negative current deflection above noise lasting ≥ 1 ms. Except for electrically-evoked currents elicited in autaptic neurons in microisland culture, current

amplitudes were measured by averaging ~500 ms of baseline and subtracting this from the average of ~500 ms around the peak and/or steady state amplitude. For Figure 4, the current ratio is measured by dividing the agonist-induced current amplitude by the KCl-induced current amplitude. For Figure 6, the baseline leak amplitude was subtracted from the peak amplitude for at least 3 traces from each cell and averaged. For autaptic experiments, cells with leak currents > 300 pA were excluded from analysis. To determine the percentage of healthy transfected neurons after agonist treatment, the number of healthy neurons was divided by the total number of transfected neurons counted in a particular culture dish and multiplied by 100. Miniature postsynaptic current frequency was calculated from 10 s of recording using MiniAnalysis software (Synptosoftware, Decatur, GA, USA). Baseline frequency was measured before application of agonist while the frequency in agonist was measured beginning ~1 s after application onset. The percentage of baseline frequency was calculated by dividing the frequency in agonist by the frequency at baseline and multiplying by 100. Data are presented in the text and figures as mean \pm SEM unless otherwise stated. Student's unpaired *t* test, ANOVA, post hoc Tukey honestly significant difference test for multiple comparisons, Tukey-Kramer method for unequal sample sizes, binomial test, and Fisher's exact test were used to determine statistical significance where described in the text. A p-value of less than 0.05 was required for significance.

Materials:

Alexa Fluor 568 was purchased from Invitrogen. Synaptophysin-YFP and TRPV1 constructs were graciously provided by Dr. Ann Marie Craig (University of

British Columbia) and Dr. David Julius (University of California San Francisco), respectively. TRPM8 has been used previously (Macpherson et al., 2006; Cruz-Orengo et al., 2008). The calcium-free culture medium used for toxicity experiments was made by the Tissue Culture Support Center at Washington University by excluding 1.8 mM calcium chloride from Neurobasal medium recipe (Brewer et al., 1993). Unless otherwise stated, all other chemicals and reagents were purchased from Sigma (St. Louis, MO, USA). When DMSO concentration in experimental solutions was $> 0.1\%$ after addition of reagents from DMSO stock solutions, control and experimental conditions were matched for final DMSO concentration.

Results

TRPV1 transfection:

We transfected neurons in our primary mass hippocampal cultures with heterologous ligand-gated ion channels to spatially and temporally control neuronal depolarization. We co-transfected a yellow fluorescent protein-tagged synaptophysin construct (Syn-YFP), which served as a marker for somata and presynaptic terminals in transfected neurons (Fig 1A, 3A). We first co-expressed the ligand-gated, nonselective cation channel TRPV1 (Fig 1A, B). Whole-cell currents (see Fig 1E, F) from YFP-positive and YFP-negative neurons in cultures co-transfected with TRPV1 showed that, at a 1:4 Syn-YFP:TRPV1 DNA ratio, no neurons expressed one construct without the other (N = 38 transfected and 19 non-transfected neurons). Assessed by YFP fluorescence, transfection efficiency was $1.1 \pm 0.3\%$ (N = 3 dishes).

To determine whether current amplitudes generated by TRPV1 were sufficiently large to cause action potentials in transfected cells, we performed cell-attached patch recordings from neurons. Cell-attached patch methods were used in order to prevent cell rupture, which would cause cytosolic mixing with internal patch pipette solution and, therefore, potentially alter the intracellular milieu and neuronal responses to environmental manipulation. Application of a saturating concentration (500 nM) of the TRPV1 agonist capsaicin (Caterina et al., 1997; Tominaga et al., 1998), which was chosen in order to maximize neuronal depolarization and potential secondary effects of channel activation, induced a pattern of 1-9 action potentials in TRPV1-expressing neurons but no action potentials in non-transfected neurons (Fig 1C, D; N = 15

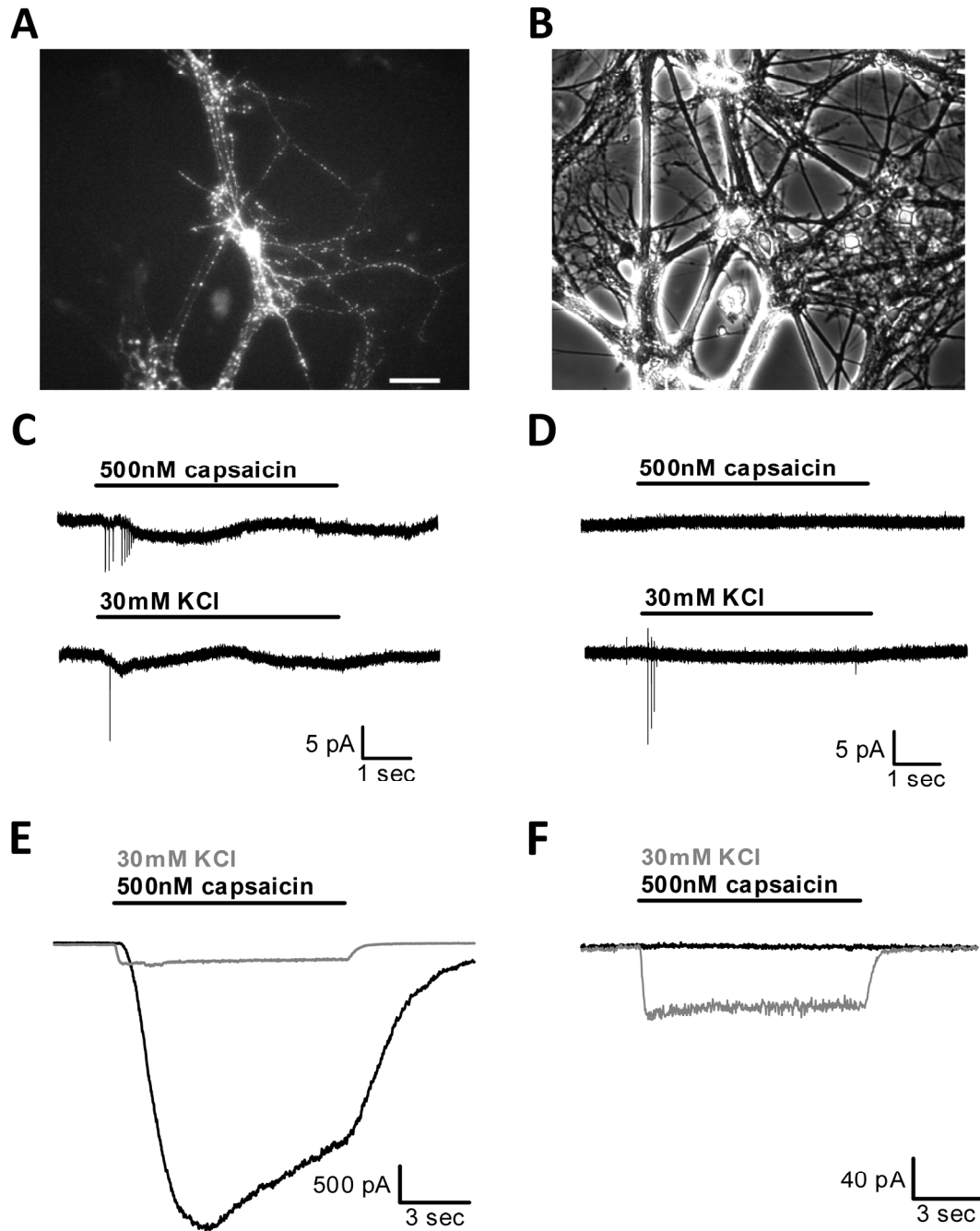


Figure 1. TRPV1-transfected neurons respond strongly to the agonist capsaicin. **A.** Fluorescence image of a neuron in mass culture transfected with synaptophysin-YFP (Syn-YFP) and TRPV1. Scale bar denotes 100 μm . **B.** Phase contrast image of the same field as A. **C.** Example of a Syn-YFP/TRPV1-transfected neuron recorded in cell-attached patch configuration in voltage-clamp mode during 5 s 500 nM capsaicin application (top trace) or 5 s 30 mM KCl application (bottom trace). Note that action potentials could be elicited by both treatments in the same cell. The suboptimal seal, evident in the slow current excursions, was characteristic of nearly all TRPV1-transfected neurons. **D.** Example of a non-transfected neuron recorded under the same conditions as

C. Note that action potentials were only elicited during KCl application. **E.** Example of a Syn-YFP/TRPV1-transfected neuron recorded in whole-cell voltage-clamp mode during 10 s acute 500 nM capsaicin or 30 mM KCl application. TRPV1-transfected neurons responded with robust currents to both capsaicin and KCl application. **F.** Example of a non-transfected neuron recorded under the same conditions as E. Non-transfected neurons never responded to capsaicin but always responded with robust KCl-induced currents.

transfected and 5 non-transfected neurons). From the same cells, 30 mM KCl application caused 1-6 action potentials in both transfected and non-transfected neurons (Fig 1C, D; N = 19 neurons). Hippocampal neurons are capable of sustaining action potential firing for periods of at least as long as our 5 s agonist application shown in Figure 1C, but spiking typically ceased soon after the start of capsaicin application. This may result from small or rapidly desensitizing capsaicin-induced currents or from very large currents that cause strong inactivation of the sodium channels necessary for spiking. This latter explanation almost certainly applied to the effects of KCl, a strong depolarizing stimulus (Hodgkin, 1947; Hodgkin and Horowicz, 1959; Moulder et al., 2006).

To determine if rapid desensitization of capsaicin-induced current is responsible for abbreviated spiking in response to capsaicin, we examined capsaicin- and KCl-elicited currents in voltage-clamped neurons. YFP-positive neurons, unlike non-transfected neurons (5.3 ± 4.4 pA; N = 4), reliably responded to the TRPV1 agonist capsaicin with robust (-1385.7 ± 574.8 pA; N = 6) currents measured via whole-cell voltage clamp (Fig 1E, F). As a comparison, we examined the response to current induced by 30 mM KCl application, which is known to clamp the membrane potential at steady state near -20 mV (Moulder et al., 2006). Non-transfected neurons responded with a KCl-induced current amplitude of -159.5 ± 62.3 pA (N = 4) while transfected neurons responded with a similar amplitude of -204.0 ± 30.8 pA (N = 6). The capsaicin current desensitized but remained large during the application period, suggesting that rapid desensitization of capsaicin-induced current is not likely to be responsible for the abbreviated spiking observed in cell-attached patch experiments. The robust sustained current in capsaicin, however, does implicate voltage-gated sodium channel inactivation

or other voltage-dependent processes as a possible explanation. We conclude that currents elicited by capsaicin in transfected neurons are similar to or larger on average than those elicited by KCl and, therefore, that 500 nM capsaicin elicits a strong yet selective depolarization in TRPV1-transfected neurons.

These observations were confirmed by neuronal responses to exogenous compounds recorded in whole-cell current-clamp, where transfected neurons produced a burst of 4-6 action potentials in response to 5 s 500 nM capsaicin and depolarized to a steady state of -4.0 ± 1.7 mV (N = 5) while non-transfected neurons did not respond to capsaicin (N = 5; Fig S1A, B; Text S1). Both transfected and non-transfected neurons produced a burst of 0 (in 1/10 cells) to 9 action potentials in response to 5 s 30 mM KCl while the membrane potential depolarized to a non-steady state value of -33.9 ± 3.4 mV during application. These results support the conclusion that acute application of capsaicin to transfected neurons can meet or exceed the strength of depolarization achieved by a calibrating KCl stimulation.

We noticed that TRPV1-transfected neurons tended to have larger basal holding currents (-177.7 ± 60.9 pA; N = 30) than control neurons (-51.1 ± 10.0 pA; N = 32; Student's unpaired *t* test, $p < 0.05$) when voltage-clamped at -70 mV. We wondered whether this observation could reflect non-ligand-gated channel opening of TRPV1. We tested this explicitly by applying the non-competitive TRPV1 antagonist ruthenium red (10 μ M), which blocked $91.8 \pm 5.3\%$ of the capsaicin-gated current in our TRPV1-transfected (500 nM capsaicin; N = 11) but caused no effect in non-transfected (N = 8) neurons (Fig 2A). When we applied 10 μ M ruthenium red to agonist-naïve, TRPV1-transfected neurons at the holding potential of -70 mV, we noticed that ruthenium red

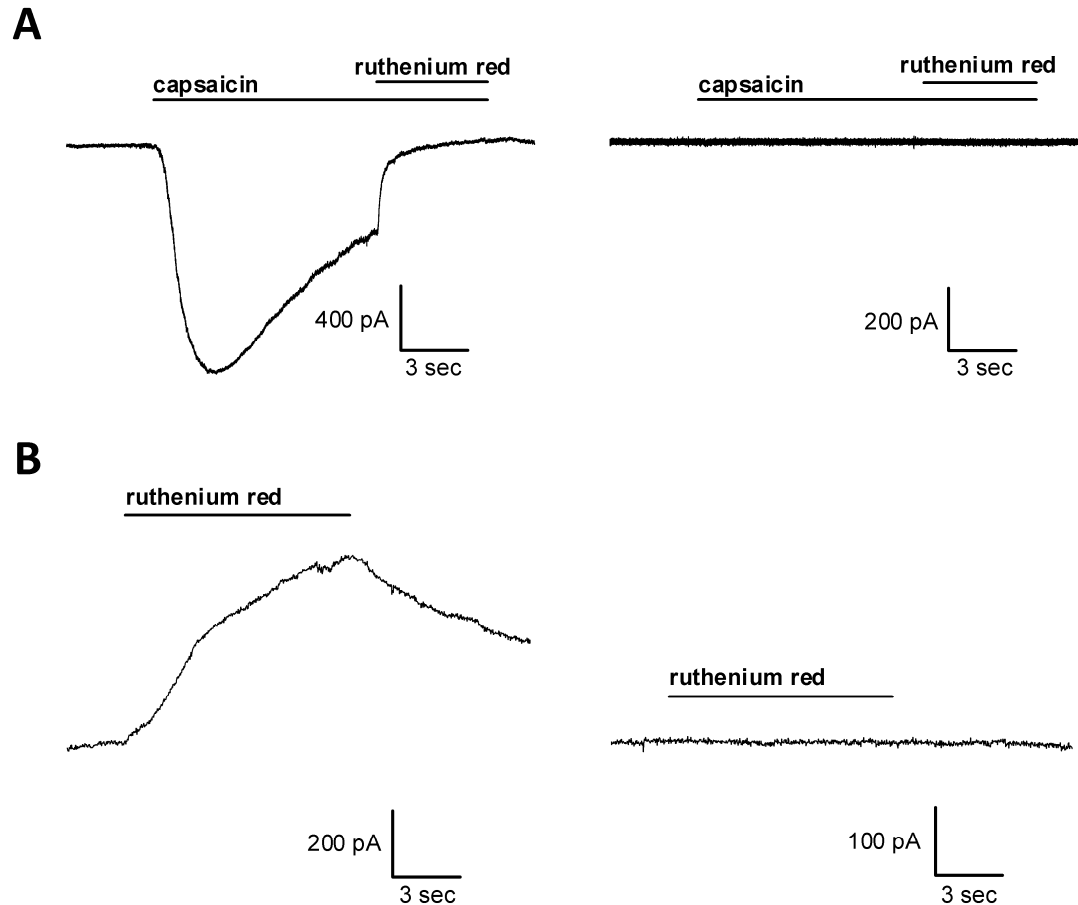


Figure 2. Capsaicin-induced and capsaicin-independent currents are blocked by ruthenium red in TRPV1-transfected neurons. **A.** Examples of TRPV1-transfected (left panel) and non-transfected (right panel) neurons exposed to acute application of 500 nM capsaicin for 15 s with 10 μ M ruthenium red added during the last 5 s. TRPV1-transfected neurons, but not non-transfected neurons, respond to capsaicin with a robust current that is blocked by ruthenium red. **B.** Examples of TRPV1-transfected (left panel) and non-transfected (right panel) neurons exposed to acute application of 10 μ M ruthenium red in the absence of capsaicin. Leaky (more than -200 pA standing inward current) TRPV1-transfected neurons reliably responded to ruthenium red while non-leaky TRPV1-transfected neurons and non-transfected neurons did not.

blocked standing inward current, especially in the leakiest (> -200 pA) TRPV1-transfected cells (Fig 2B). Ruthenium red blocked $12.3 \pm 3.1\%$ ($N = 15$) of standing inward current in TRPV1-transfected neurons while not affecting non-transfected neurons ($1.5 \pm 1.8\%$; $N = 9$; Student's unpaired t test, $p < 0.05$). This suggests that at least some TRPV1-transfected neurons exhibit capsaicin-independent channel openings, even in defined recording media, which could be detrimental to the goal of controlling TRPV1-mediated depolarization.

TRPM8 transfection:

We also transfected cultured hippocampal neurons with the ligand-gated nonselective cation channel TRPM8 (Fig 3A, B). At a 1:3 Syn-YFP:TRPM8 DNA ratio, we confirmed that transfected neurons responded to acute application of the TRPM8 agonist menthol at a saturating concentration ($100 \mu\text{M}$; $N = 38$) (McKemy et al., 2002; Liu and Qin, 2005), once again chosen to maximize effects of channel activation, while non-transfected cells did not respond ($N = 10$). Similar to the experiments above with TRPV1, transfection efficiency was $1.2 \pm 0.4\%$ as assessed by YFP fluorescence ($N = 3$ dishes).

YFP-positive neurons recorded in the cell-attached patch configuration responded with a pattern of 1-7 action potentials during $100 \mu\text{M}$ menthol application, as they did during KCl application (Fig 3C; $N = 13$ transfected neurons). These action potential responses were similar to those seen in TRPV1-transfected neurons (Fig 1C), and non-transfected neurons responded to KCl with 1-8 action potentials but did not respond to menthol (Fig 3D; $N = 26$ non-transfected neurons). When we applied these exogenous

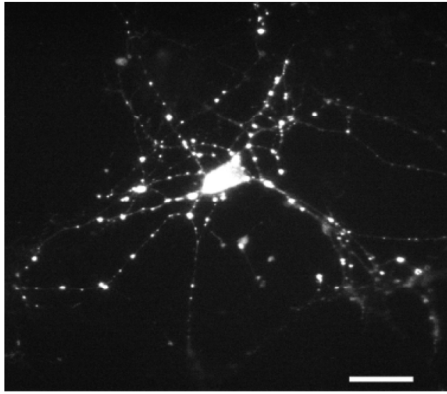
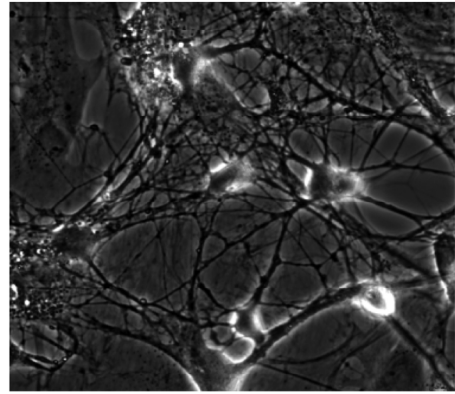
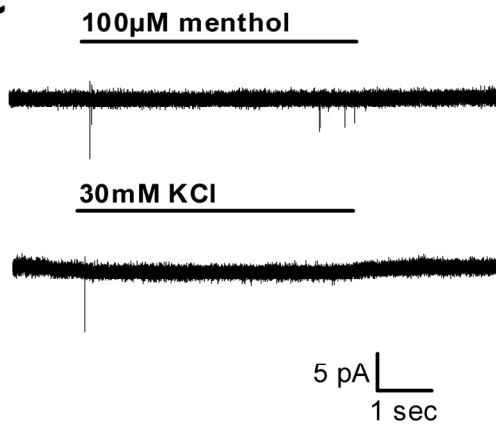
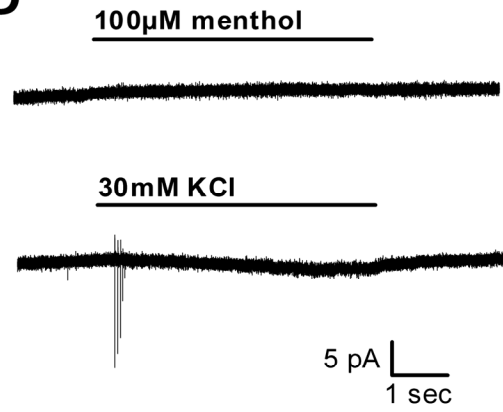
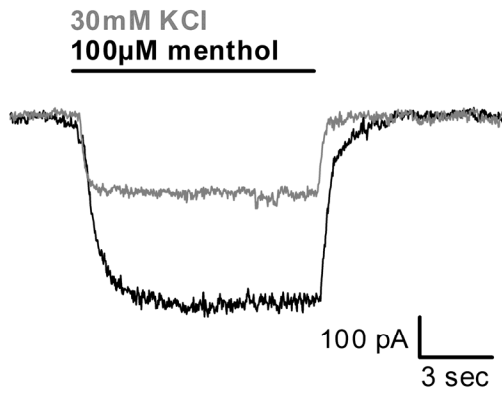
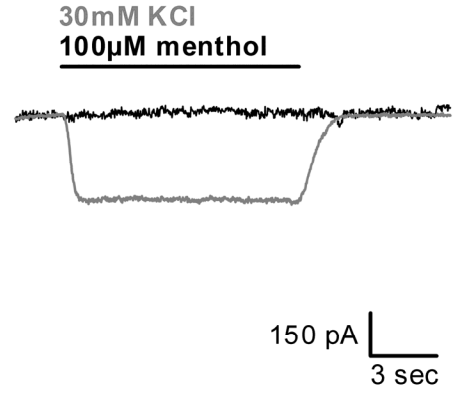
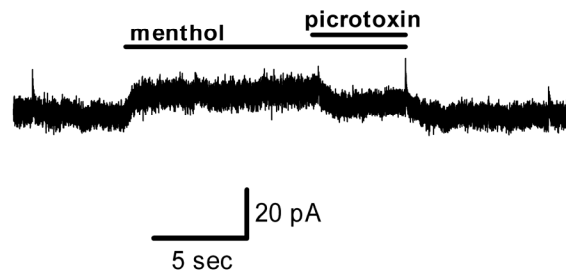
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Figure 3. Menthol induces large depolarizing currents in TRPM8-transfected neurons. **A.** Fluorescence image of a neuron in mass culture transfected with synaptophysin-YFP (Syn-YFP) and TRPM8. Scale bar denotes 50 μm . **B.** Phase contrast image of the same field as A. **C.** Example of a Syn-YFP/TRPM8-transfected neuron recorded in cell-attached patch configuration in voltage-clamp mode during 5 s 100 μM menthol application (top trace) or 5 s 30 mM KCl application (bottom trace). Note that action potentials were elicited by both treatments in the same cell. **D.** Example of a non-transfected neuron recorded under the same conditions as C. Note that action potentials were only elicited during KCl application. **E.** Example of a Syn-YFP/TRPM8-transfected neuron recorded in whole-cell voltage-clamp during 10 s acute 100 μM menthol or 30 mM KCl application. TRPM8-transfected neurons responded with robust currents to both menthol and KCl application. **F.** Example of a non-transfected neuron recorded under the same conditions as E. Non-transfected neurons did not respond to menthol but always responded with robust currents to KCl at the holding potential of -70 mV. **G.** A non-transfected neuron recorded in whole-cell voltage clamp held at 0 mV during 15 s acute application of 100 μM menthol with 100 μM picrotoxin added during the last 5 s. Note that picrotoxin decreased the current gated by menthol.

compounds while recording from TRPM8-transfected neurons in current-clamp, we again observed bursts of 1-6 action potentials and a steady state membrane potential of -13.1 ± 6.2 mV elicited by 5 s 100 μ M menthol application (N = 6; Fig S1C; Text S1). Non-transfected neurons did not respond to menthol application (N = 5; Fig S1D). In these experiments, 30 mM KCl consistently elicited 1-3 action potentials in all cells and a membrane potential after 5 s, though not yet at steady state, of -37.1 ± 1.9 mV. TRPM8-transfected neurons exhibited large inward currents measured in voltage-clamp during acute menthol application (-396.4 ± 69.6 pA; N = 28) while YFP-negative neurons did not respond with inward current (23.9 ± 20.1 pA; N = 10) at -70 mV (Fig 3E, F). As seen with TRPV1-transfected neurons, these menthol-induced currents in TRPM8-transfected neurons were similar to or larger than currents induced by 30 mM KCl (-153.4 ± 27.8 pA; N = 8 transfected neurons; -326.6 ± 96.2 pA; N = 10 non-transfected neurons). We conclude that both TRPV1 and TRPM8 generate sufficiently large currents in transfected hippocampal neurons to cause strong, sustained (for several seconds) depolarization with acute agonist application, equivalent to or larger than the effects of 30 mM KCl application.

Standing inward current in TRPM8-transfected neurons (-44.7 ± 16.9 pA; N = 32) was not significantly different from non-transfected cells (-57.2 ± 36.5 pA; N = 8), suggesting that TRPM8 does not suffer from the same non-ligand-gated channel opening as TRPV1. However, in some non-transfected cells held at 0 mV, we observed a small sustained outward current in the presence of 100 μ M menthol (13.1 ± 1.3 pA; Fig 3G; N = 8). This current was observed even in the presence of 0.5 μ M TTX and changed direction appropriately with alterations in the chloride gradient (Fig S2; Text S1). This

latter result excludes the possibility that the current is mediated by endogenous TRPM8 in non-transfected neurons, and no published evidence currently indicates endogenous TRPM8 expression in rodent hippocampus. A previous report suggests, however, that menthol can directly gate GABA_A receptor-mediated chloride currents in hippocampal neurons (Zhang et al., 2008). Picrotoxin (100 μM), a noncompetitive GABA_A receptor antagonist, reduced the outward current in non-transfected neurons by $85.4 \pm 12.9\%$ (Fig 3G; N = 8 neurons). Therefore, these data provide evidence that menthol may weakly gate endogenous GABA_A receptors in cultured neurons.

Prolonged channel activation:

To control neuronal activity and evaluate the effects on development, synaptic function, or network activity, it may be desirable to depolarize neurons for long periods of time. For this reason, we activated TRPV1 and TRPM8 in cultured neurons for 4 hr to determine if depolarization was sustained throughout the period of agonist exposure. We chose to activate these channels for hours, instead of a shorter time period, to account for slow forms of channel desensitization or inactivation that may be induced (i.e. by cellular adaptation). These slower changes may be important considerations for studies using heterologous channels *in vivo* or for studies of neuronal circuit activity that utilize changes in activity of subsets of neurons for long periods. To activate TRPV1, we added 500 nM capsaicin to the culture media in the presence of D-APV (25 μM) and NBQX (1 μM) while the neurons remained at 37 °C in an atmospherically-controlled incubator. Similarly, TRPM8-transfected cultures were exposed to 100 μM menthol under the same conditions.

At the end of incubation, neurons were recorded in the continued presence of agonist, and agonist-induced currents were determined by removing the agonist with a fresh saline wash and measuring the change in current amplitude. This amplitude was then compared to the current amplitude of an acute application of 30 mM KCl in the same cell (Fig 4A, B). Compared to brief (0-30 min) application of agonist, normalized current amplitudes for both TRPV1- and TRPM8-transfected neurons were significantly decreased after 4 hr application of their respective agonists (Fig 4C, D). The ratio of capsaicin current to KCl current in TRPV1-transfected neurons decreased from 3.04 ± 0.73 (N = 32 neurons treated acutely with capsaicin) to 0.32 ± 0.08 (N = 11 neurons treated 4 hr with capsaicin; Student's unpaired *t* test, $p < 0.05$). The ratio of menthol current to KCl current in TRPM8-transfected neurons decreased from 8.28 ± 3.24 (N = 7 neurons treated acutely with menthol) to 1.56 ± 0.40 (N = 10 neurons treated 4 hr with menthol; Student's unpaired *t* test, $p < 0.05$). The inward current present after 4 hr, however, was still comparable to the current induced by acute 30 mM KCl. In both acute and prolonged protocols, the average KCl-normalized menthol current in TRPM8-transfected neurons trended toward being larger than the corresponding normalized capsaicin current in TRPV1-transfected cells. In summary prolonged agonist application produced sustained, effective depolarization for both channels, although the amplitude was reduced with time.

The smaller average agonist-gated current after prolonged exposure probably results partly from receptor desensitization. However, it is also possible that strong, prolonged channel activation is toxic to neurons, and the smaller average currents arise as a result of selective survival of cells with weaker channel expression and smaller

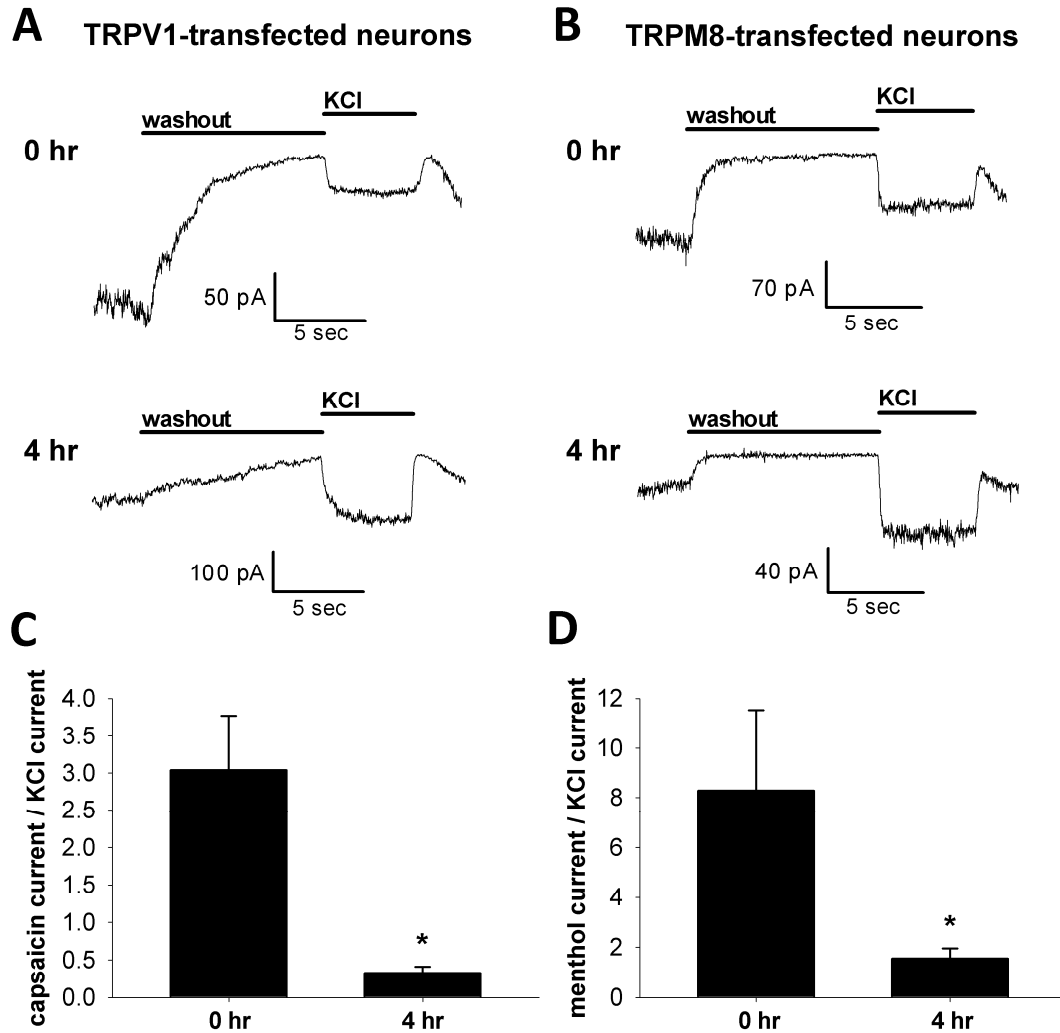


Figure 4. Agonist-induced currents in both TRPV1- and TRPM8-transfected neurons are decreased after prolonged exposure. **A.** Examples of TRPV1-transfected neurons exposed to bath application of 500 nM capsaicin, 25 μ M D-APV, and 1 μ M NBQX acutely (top panel) or for 4 hr (bottom panel) prior to recording in whole-cell voltage clamp. Capsaicin was retained in the bath recording solution. After the whole-cell recording was established, capsaicin was briefly removed by perfusing fresh recording saline for 10 s before 5 s application of 30 mM KCl. **B.** Examples of TRPM8-transfected neurons exposed to bath application of 100 μ M menthol, 25 μ M D-APV, and 1 μ M NBQX acutely (top panel) or for 4 hr (bottom panel) prior to recording in whole-cell voltage clamp. Similar to **A**, menthol was added to the bath recording solution. Menthol was removed by perfusing fresh, menthol-free recording saline for 10 s before 5 s application of 30 mM KCl. **C.** The average ratio of capsaicin current amplitude to KCl current amplitude was calculated for acute and 4 hr applications of capsaicin. Error bars represent SEM. The ratio of capsaicin to KCl current is significantly decreased after 4 hr capsaicin exposure (Student's unpaired *t* test, $p < 0.05$). **D.** Similar to **C** except the ratio of menthol to KCl currents in TRPM8-transfected neurons is depicted. Error bars represent SEM, and the menthol to KCl current ratio is significantly decreased after 4 hr exposure to menthol (Student's unpaired *t* test, $p < 0.05$).

resulted in many unsuccessful electrophysiological recordings of cells with abnormally swollen morphology. To determine more systematically whether prolonged channel activation affects neuronal health, mass cultures were transfected with Syn-YFP control DNA with or without TRPV1 or TRPM8. Neurons were assigned a binary (“healthy” or “unhealthy”) survival designation by a naïve observer using cell appearance under phase contrast optics. Healthy neurons had a phase-bright soma and thin neurites. Unhealthy neurons had distorted or swollen somata and neurites (Fig 5A). In some cases, classification was verified by patch-clamp recordings; membrane seals were never possible on neurons designated unhealthy.

Neurons were assessed for health after 4 hr of channel activation using the same treatment protocol as the electrophysiology experiments in Figure 4. It is possible that transfection alone caused some baseline toxicity; however, after 4 hr of capsaicin exposure, the percentage of healthy TRPV1-transfected neurons was significantly decreased from control cultures transfected with only Syn-YFP (Fig 5B; ANOVA, $p < 0.01$; post hoc Tukey honestly significant difference test, $p < 0.05$; $N = 5$ dishes per condition with each dish representing 19.9 ± 1.3 transfected neurons). In contrast, a 4 hr menthol challenge to TRPM8-transfected neurons caused no significant change in the percentage of healthy neurons relative to sibling cultures transfected with Syn-YFP (Fig 5C; $N = 5$ dishes per condition with each dish representing 22.3 ± 2.4 transfected neurons).

Calcium influx may differ between transfected neurons due to differences in TRPV1 and TRPM8 calcium permeability (Caterina et al., 1997; McKemy et al., 2002).

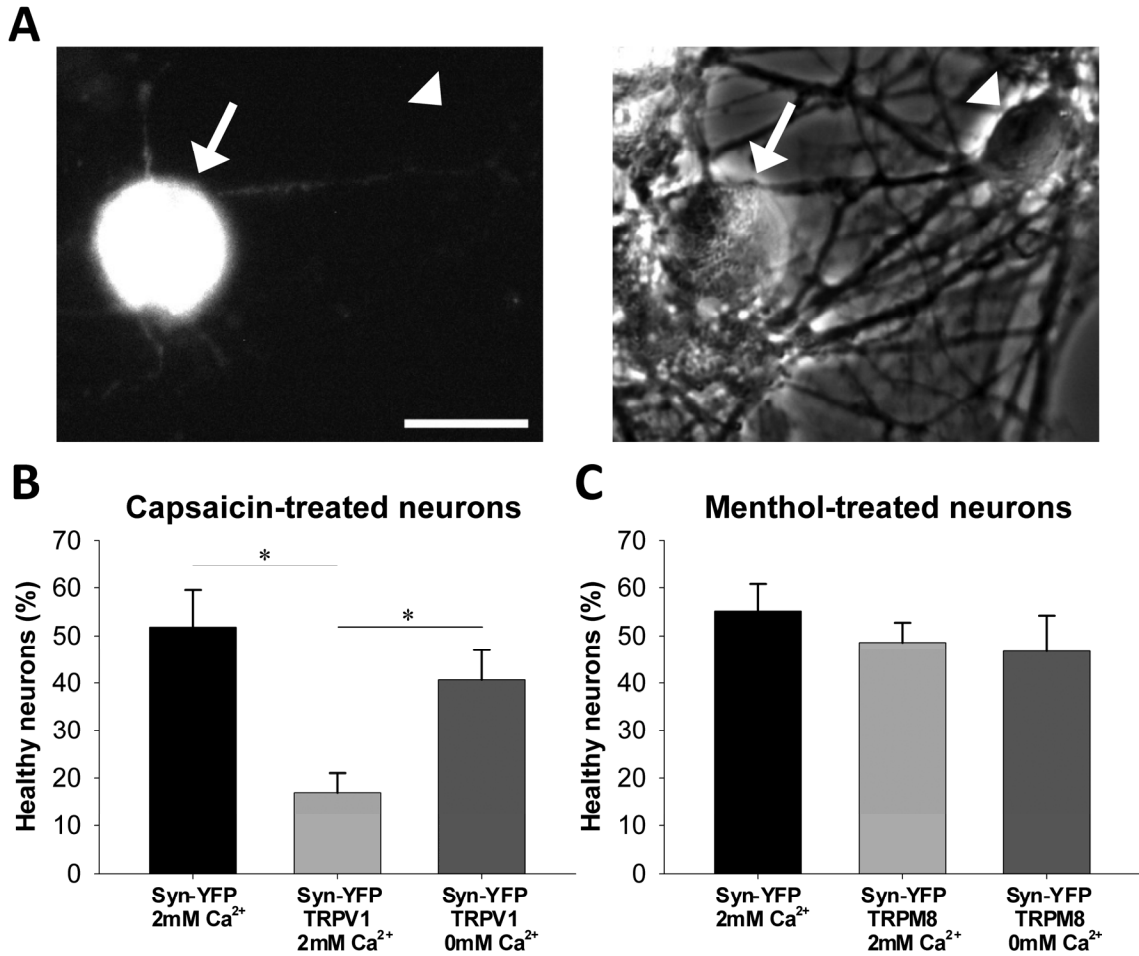


Figure 5. Agonist-induced toxicity in transfected neurons. A. An example TRPV1-transfected neuron designated “unhealthy” (arrow) in the same field as a “healthy” non-transfected neuron (arrowhead). Syn-YFP fluorescence (left panel) and phase contrast (right panel) images are shown. Note the swollen cell body of the unhealthy cell. Scale bar represents 30 μm . **B.** Transfected neurons treated 4 hr with 500 nM capsaicin, 25 μM D-APV, and 1 μM NBQX in culture media with (2 mM) or without (0 mM plus 500 μM EGTA) calcium were assessed as “healthy” or “unhealthy” by a naïve observer. Significantly fewer TRPV1-transfected neurons treated in calcium were healthy than either YFP-transfected neurons treated in calcium or TRPV1-transfected neurons treated without calcium (ANOVA, $p < 0.01$; post hoc Tukey test, $p < 0.05$). **C.** Transfected neurons treated 4 hr with 100 μM menthol, 25 μM D-APV, and 1 μM NBQX in culture media with or without calcium were assessed as “healthy” or “unhealthy” as in B. There was no significant change in health of TRPM8-transfected neurons compared to YFP-transfected neurons regardless of whether or not they were exposed to extracellular calcium. Error bars represent SEM.

Because calcium influx is known to contribute to some forms of cellular toxicity (Choi, 1987; Stout et al., 1998; Mattson et al., 2000), we wished to determine if toxicity in TRPV1-transfected neurons after prolonged channel activation was due to calcium influx. We assayed toxicity after 4 hr of agonist exposure in calcium-free extracellular media supplemented with 500 μ M of the calcium chelator ethylene glycol-bis (β -aminoethyl ether)-*N, N, N', N'*-tetraacetic acid (EGTA). Calcium-free media protected TRPV1-expressing neurons exposed to 500 nM capsaicin compared to those in calcium-containing media (Fig 5B); neurons exposed to capsaicin in calcium-free media appeared similar to control neurons not expressing TRPV1. Survival of TRPM8-expressing neurons exposed to 100 μ M menthol in calcium-free media did not differ from either Syn-YFP-transfected controls or from TRPM8-transfected neurons treated in calcium-containing media (Fig 5C). This suggests that calcium influx during TRPV1 activation, but not during TRPM8 activation, is toxic to neurons.

Effects of channel expression on synaptic transmission:

For the utility of heterologous channels to be realized, it is important to ensure that channel introduction alone does not alter endogenous neuronal function outside of intended experimental manipulations. To determine if expression of heterologous ion channels alters synaptic properties of cultured hippocampal neurons, we transfected neurons in microisland culture with TRPV1 or TRPM8. Microisland cultures, unlike mass cultures, contain isolated “islands” of astrocytes with neurons plated on top (Fig 6A). Measuring the autaptic responses of single neurons that synapse only onto themselves is a useful method for studying synaptic efficacy in the absence of

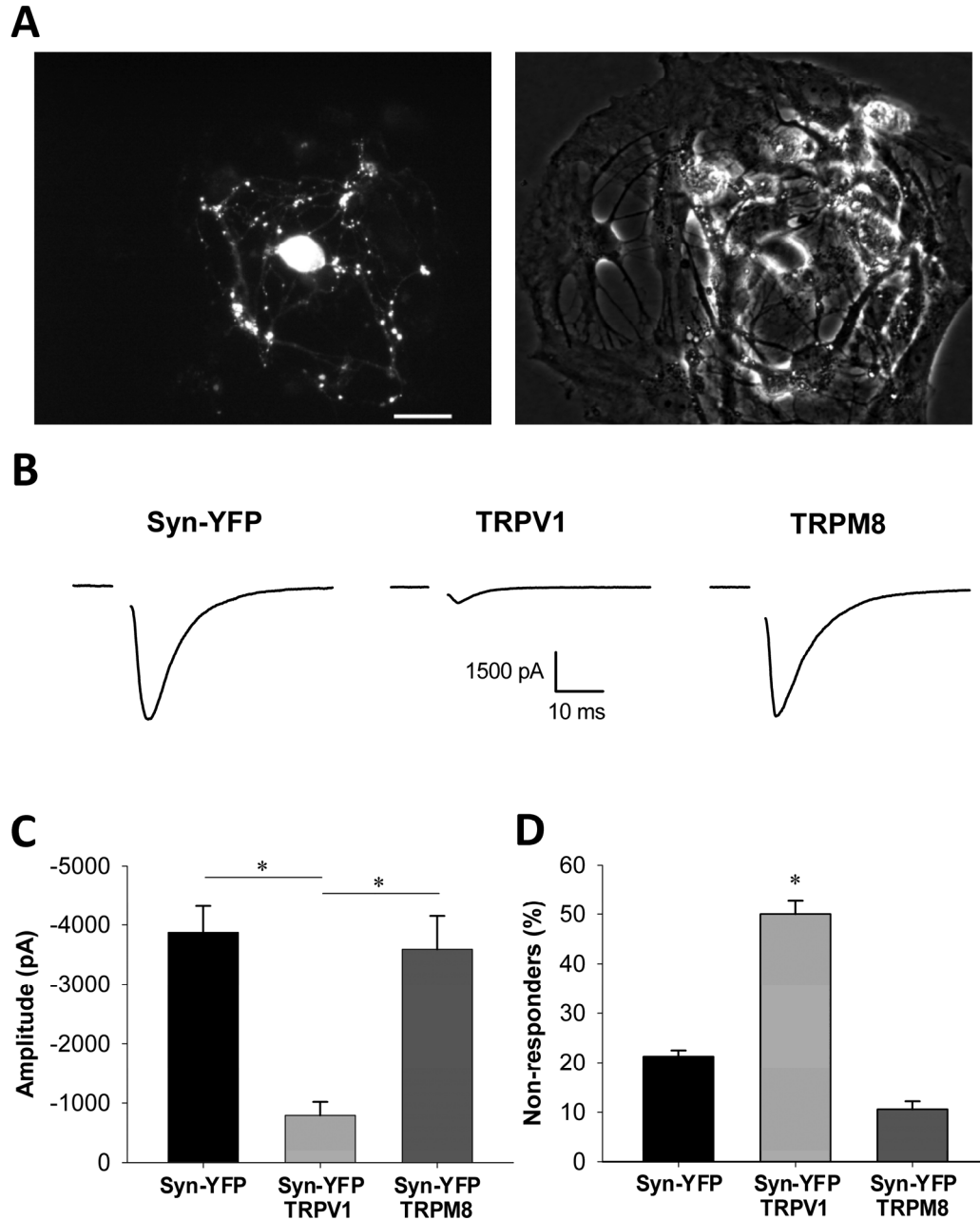


Figure 6. TRPV1 transfection, but not TRPM8 transfection, alters synaptic transmission. **A.** Fluorescence (left panel) and phase contrast (right panel) images of a single-neuron (autaptic) island in microisland culture transfected with synaptophysin-YFP (Syn-YFP). Scale bar denotes 50 μ M. **B.** Representative examples of excitatory postsynaptic currents (EPSCs) evoked in transfected autaptic neurons 1 day after transfection. Stimulus artifact has been blanked in each trace for clarity. Note that TRPV1-transfected neurons produced EPSCs with smaller amplitudes than TRPM8-transfected neurons or Syn-YFP-transfected controls. **C.** Quantification of average PSC amplitudes for transfected autaptic neurons. Error bars represent SEM. TRPV1-transfected neurons produced significantly smaller evoked PSCs than Syn-YFP-transfected or TRPM8-transfected neurons (ANOVA, $p < 0.01$; Tukey-Kramer method, p

< 0.01). **D.** Percentage of transfected autaptic neurons that did not respond to electrical stimulation with a detectable PSC. When non-responders were analyzed as “failures” from a binomial distribution, the percentage of TRPV1-transfected neurons that successfully responded to stimulation was significantly decreased from YFP-transfected controls (binomial test, $p < 0.01$). Error bars represent SEM based on the binomial distribution.

polysynaptic complications (Mennerick et al., 1995). Only a minority of island cultures contains a solitary autaptic cell. Because of this low percentage, combined with low transfection efficiency, we pooled synaptic responses from glutamatergic and GABAergic cells. We used KCl internal pipette solution to create a driving force of approximately 70 mV for both excitatory postsynaptic currents (EPSCs) and inhibitory postsynaptic currents (IPSCs) at the holding potential of -70 mV.

TRPV1-transfected autaptic neurons exhibited action potential-evoked autaptic PSC amplitudes (-790.9 ± 228.3 pA; N = 9) that were significantly decreased from Syn-YFP-transfected controls (-3870.9 ± 444.1 pA; N = 28; Fig 6B, C; ANOVA, $p < 0.01$; Tukey-Kramer method, $p < 0.01$). TRPM8-transfected autaptic neurons, however, exhibited PSC amplitudes of -3590.8 ± 557.3 pA (N = 17), which were comparable to Syn-YFP-transfected controls (Fig 6B, C). In addition to smaller amplitudes of evoked PSCs, we observed that more cells in TRPV1-transfected cultures exhibited no PSC in response to action potential stimulation. In autaptic neurons, 50% of TRPV1-transfected neurons failed to produce a response, which was significantly more than Syn-YFP-transfected controls (21%; Fig 6D; binomial test, $p < 0.01$; N = 18 TRPV1-transfected and 33 Syn-YFP-transfected autaptic neurons recorded in microisland culture). With a failure rate of 11%, TRPM8-transfected neurons were not significantly different from controls (N = 19 TRPM8-transfected neurons). This phenomenon appears to be an extension of the observation of smaller evoked PSCs in TRPV1-transfected neurons. In summary TRPV1 transfection, in the absence of added ligand, depressed evoked synaptic transmission in autaptic hippocampal neurons whereas TRPM8 transfection had no effect.

One possible reason that TRPM8 does not interfere with synaptic transmission is that neurons may not express the channel at synaptic sites. TRPM8 seems targeted robustly to the somatodendritic compartment as evidenced by currents recorded from the soma (Fig 3E). Whether the channel is targeted to presynaptic terminals is less clear. If it is, axon terminal expression of TRPM8 could also be useful for probing functional connections between neurons of disparate brain regions. We used a functional test of presynaptic TRPM8 expression by investigating if TRPM8 drives synaptic neurotransmitter release independent of action potential propagation. We recorded from non-transfected target cells apposed to Syn-YFP-positive puncta (Fig 7A). We applied 100 μ M menthol to neurons in the presence of 0.5 μ M TTX to block action potential-driven transmitter release. If agonist-induced miniature EPSCs (mEPSCs) were not observed at a holding potential of -70 mV when using a cesium methanesulfonate internal pipette solution, then the holding potential was switched to 0 mV to record miniature IPSCs (mIPSCs). Baseline mPSC frequency, which presumably arose from mEPSCs and mIPSCs from both transfected and non-transfected presynaptic terminals, was highly variable in postsynaptic targets of both TRPM8-transfected (0.1-119.8 Hz) and control (0.4-108.0 Hz) neurons. Therefore, we expressed the changes in frequency that occurred during menthol application as a percentage of baseline frequency. Acute menthol application to TRPM8-transfected neurons (N = 26) increased mPSC frequency in the postsynaptic cell to $1090.4 \pm 532.1\%$ of baseline frequency from before menthol application. This was significantly increased from non-transfected neurons or neurons transfected with only Syn-YFP (N = 13), which produced a mPSC frequency in menthol of $104.1 \pm 6.3\%$ of baseline frequency (Fig 7B, C; Fisher's exact test, $p < 0.005$). We

conclude that it possible to depolarize presynaptic terminals directly with menthol, which suggests that functional TRPM8 protein is expressed on or very near axon terminals.

Since synaptic transmission is depressed in TRPV1-transfected neurons (Fig 6), we tested whether TRPV1 is expressed near synaptic sites and whether presynaptic release is hindered. The baseline mPSC frequency, like with TRPM8 transfection, varied greatly in the postsynaptic targets of control (0.5-93.2 Hz) and, less so, TRPV1-transfected (1.0-35.1 Hz) neurons, although the mean baseline mPSC frequency (9.5 ± 7.1 Hz and 15.3 ± 5.2 Hz, respectively) did not differ significantly. When 500 nM capsaicin was applied to Syn-YFP-positive puncta of TRPV1-transfected neurons apposed to non-transfected neurons in the presence of 0.5 μ M TTX, the mPSC frequency increased to $1016.8 \pm 323.2\%$ of baseline frequency (N = 7; Fig 7D). This significantly differed from control neurons not in contact with transfected neurons (N = 9), for which capsaicin induced a mPSC frequency of only $121.3 \pm 34.7\%$ of baseline frequency (Fig 7D; Fisher's exact test, $p < 0.01$). Like with TRPM8-transfected neurons, this suggests that functional TRPV1 protein is expressed on or very near axon terminals.

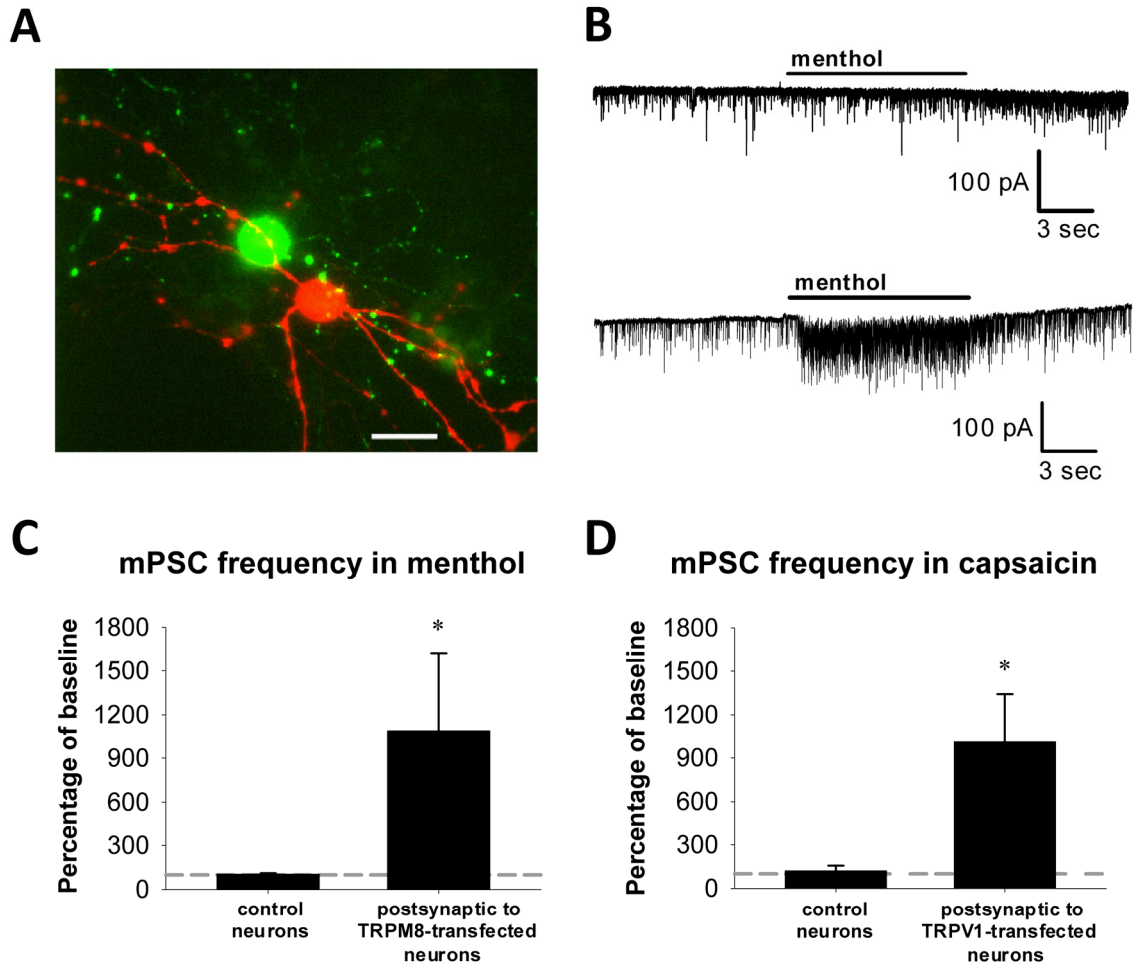


Figure 7. Agonist treatment in 0.5 μ M tetrodotoxin (TTX) increases presynaptic transmitter release in transfected neurons. **A.** Fluorescence image of a TRPM8-transfected neuron (green) forming presumed presynaptic contacts onto a postsynaptically-recorded cell (red) filled with Alexa Fluor 568, which was included in the recording pipette. Scale bar denotes 50 μ M. **B.** Example of miniature excitatory postsynaptic currents (mEPSCs) recorded from non-transfected postsynaptic partners of TRPM8-transfected neurons (bottom panel) or from non-transfected neurons not in contact with transfected neurons (top panel) in 0.5 μ M TTX. During 100 μ M menthol application, mEPSC and mIPSC frequency increased in neurons postsynaptic to TRPM8-transfected neurons but not in control neurons. **C.** Quantification of the mPSC frequency during 100 μ M menthol application compared to the baseline frequency (dashed line). Error bars represent SEM. In neurons postsynaptic to TRPM8-transfected neurons, but not in control neurons, the mPSC frequency increased significantly in menthol (Fisher's exact test, $p < 0.005$). **D.** Quantification of the mPSC frequency during 500 nM capsaicin application compared to baseline frequency (dashed line) in neurons postsynaptic to TRPV1-transfected neurons or to control neurons. Error bars represent SEM. In neurons postsynaptic to TRPV1-transfected neurons, but not to non-transfected neurons, the mPSC frequency in capsaicin increased significantly (Fisher's exact test, $p < 0.01$).

Discussion

In this study we compared transfection of cultured hippocampal neurons with TRPV1 or TRPM8 nonselective cation channels, which both allow specific activation of a small subset of neurons within the neuronal network (Fig 1, 3). Although TRPM8 activation mediates similar current densities to TRPV1 (Fig 4), TRPV1 has larger secondary effects on cell function, including interference with endogenous synaptic transmission in the absence of exogenous agonist stimulation (Fig 6) and overt calcium-dependent toxicity with agonist exposure (Fig 5). Our results highlight substantial differences between two related channels and suggest that care is needed in investigating secondary effects of heterologous channels before employing them as tools.

Our observations suggest that transfection of TRPV1 alone, in the absence of capsaicin stimulation, has important effects on neuronal function. Although we did not detect overt cell loss or swelling of capsaicin-naïve TRPV1-transfected cells in estimates of transfection efficiency 24 hr following transfection, cell-attached patch recordings from TRPV1-transfected cells exhibited markedly more seal instability (Fig 1C) and spontaneous rupture compared with TRPM8-transfected cells (Fig 3C). In addition, evidence suggests that at least some TRPV1 channels are activated in the absence of exogenous capsaicin application (Fig 2). Seal instability could represent an early sign of toxicity from low-probability non-ligand-gated channel openings and/or from low-level presence of endogenous activators in the culture medium (e.g. pH, lipid compounds, or heat) in the absence of capsaicin (Tominaga and Tominaga, 2005). Since the effects of TRP channel agonists are enhanced at more depolarized membrane potentials (Voets et al., 2004), it is possible that spontaneous activity may shift the heat sensitivity of TRPV1

enough to cause a persistent leak current while in the incubator. However, this cannot explain the persistent current measured electrophysiologically (Fig 2) since all recordings were performed at room temperature. Alternatively, TRPV1 overexpression itself (in the absence of channel activity) could promote these signs of compromised membrane integrity.

These same explanations (low-level channel openings or channel overexpression itself) may account for the strong synaptic depression observed in TRPV1-transfected neurons in the absence of capsaicin stimulation (Fig 6). Since these synaptic changes were measured in autaptic neurons, they could be presynaptic or postsynaptic. Because action potential-independent transmitter release from TRPV1-transfected presynaptic terminals appeared intact (Fig 7), autaptic depression may represent a postsynaptic change, but it is also possible that TRPV1 expression disrupts presynaptic action potential propagation (e.g. as a result of a leaky axonal membrane) and/or action potential coupling to transmitter release. We have previously found that neuronal depolarization in cultured hippocampal neurons leads to a homeostatic decrease in EPSC amplitude and mEPSC frequency (Moulder et al., 2004). It is possible that low-level TRPV1 channel openings induce a similar phenomenon, although we might have expected stronger evidence of reduced mPSC frequency from TRPV1-transfected neurons (Fig 7). One possibility is that contributions from non-transfected presynaptic cells reduced the sensitivity of this experiment to detect synaptic depression. The autaptic experiments did not suffer this limitation. Regardless of the mechanism, this autaptic depression in the absence of added exogenous agonist is a poorly controlled, unintended consequence of TRPV1 overexpression and represents a major disadvantage of heterologous TRPV1

expression.

Prolonged capsaicin-induced TRPV1 activation led to increased overt signs of cell toxicity (Fig 5B). This again contrasted with TRPM8-transfected cells (Fig 5C) despite similar normalized depolarizing currents (Fig 4). By activating TRPV1 in the absence of extracellular calcium, we determined that the toxicity most likely results from calcium influx into the cell (Fig 5B). Since ionotropic glutamate receptor blockers were present during these experiments, toxicity cannot be attributed to calcium influx through NMDA receptors via indirect increases in network excitability. Thus, the high calcium permeability of the TRPV1 channel relative to TRPM8 (Caterina et al., 1997; McKemy et al., 2002) is likely a strong contributor to toxicity. These results are akin to higher toxicity of calcium-permeable NMDA receptor activation relative to AMPA receptor activation (Choi, 1987; Iino et al., 1990; Goldberg and Choi, 1993; Spruston et al., 1995). It is possible that activation-dependent pore dilation, which occurs in TRPV1 (Meyers et al., 2003; Chung et al., 2008) but not TRPM8 (Chen et al., 2009), also participates in the stronger toxicity induced by capsaicin treatment. Using saturating concentrations of agonists for these treatments may have exacerbated toxicity, so it is possible that lower concentrations could be titrated to produce significant depolarizations without concurrent toxicity or other secondary effects.

Together, the experiments in this study suggest serious disadvantages of TRPV1 overexpression. To complicate matters, recent evidence also suggests that functional TRPV1 is expressed endogenously in the central nervous system (e.g. the hippocampus) in addition to its well-known expression in the peripheral nervous system (Toth et al., 2005; Cristino et al., 2008; Gibson et al., 2008). Use of heterologous channels for

selective stimulation obviously requires the demonstrated absence of contributions from endogenous channels. In our experiments, we never observed capsaicin-induced effects in non-transfected neurons (e.g. Fig 1D, 1F, 2, 7D, and S1B), suggesting that endogenous receptor had a negligible contribution. This lack of detection of endogenous TRPV1 activation may be due to differences in expression in cultured neurons or to experimental manipulations that, for whatever reason, did not measure the effects of endogenous channel activation.

TRPM8 appears to be a much better candidate than TRPV1 for studies utilizing heterologous channel expression. TRPM8 activation produced robust currents (Fig 3, 4) but induced less toxicity than TRPV1 (Fig 5C) and did not alter baseline synaptic transmission (Fig 6). Additionally, we have demonstrated that TRPM8, like TRPV1, can be used to manipulate local presynaptic function. Agonist application to transfected neurites in the absence of action potentials increased miniature PSC frequency in the postsynaptic cell (Fig 7). This suggests that agonist application stimulates calcium entry into presynaptic terminals through the TRP channel and/or through local voltage-gated calcium channels to cause neurotransmitter release. Using menthol as a TRPM8 agonist, however, has its caveats; menthol induced a small current mediated by GABA_A receptors (Fig 3G, S2). This current, however, was easily reduced by applying the GABA_A antagonist picrotoxin, and other agonist choices for TRPM8 activation exist (McKemy et al., 2002). We conclude that TRPM8 is superior for most purposes and has fewer caveats than TRPV1.

Our study has shown how two related ligand-gated ion channels, TRPV1 and TRPM8, behave differently when expressed in the same neuronal preparation. Although

we have shown that these channels, but especially TRPM8, are useful tools for controlling neuronal activity, this study also emphasizes the care needed in characterizing the nature of a newly introduced tool like heterologous channel expression. In the future, it will be important to ensure that other heterologous channels or proteins used to manipulate neuronal activity behave as predicted, especially after long periods of activation. Controlling neuronal activity in subsets of neurons within a network will facilitate studies of neuronal development, functional anatomy, synaptogenesis, and synaptic plasticity as well as provide a framework for studies manipulating neuronal populations *in vivo*.

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Text S1

Materials and Methods

Electrophysiology:

Whole-cell current-clamp experiments were performed on DIV 12-13 neurons in mass culture 1 day after transfection with TRPV1 or TRPM8. External recording media contained 138 mM NaCl, 4 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, 10 mM HEPES, 25 μM D-APV, and 1 μM NBQX (pH = 7.25). Internal pipette solution contained 140 mM K-gluconate, 4 mM NaCl, 0.5 mM CaCl₂, 5 mM EGTA, and 10 mM HEPES at a pH of 7.25. Recording electrode resistances of 6-9 MΩ and a 10 kHz low-pass filter were used for these experiments. The membrane potential of each cell was manually adjusted to -65 mV by injecting bias current before the start of recording.

For voltage-clamp experiments measuring small menthol-induced currents in non-transfected neurons, methods from the main text were utilized except that an internal pipette solution of 130 mM cesium chloride, 3 mM NaCl, 0.5 mM CaCl₂, 5 mM EGTA, and 10 mM HEPES (pH of 7.25) was used as described in addition to the solution with 130 mM cesium methanesulfonate, 4 mM NaCl, 0.5 mM CaCl₂, 5 mM EGTA, and 10 mM HEPES (pH of 7.25). Also, the external recording media was as described above except that 0.5 μM tetrodotoxin was added. The multi-barrel perfusion system described in the main text was utilized for locally applying compounds.

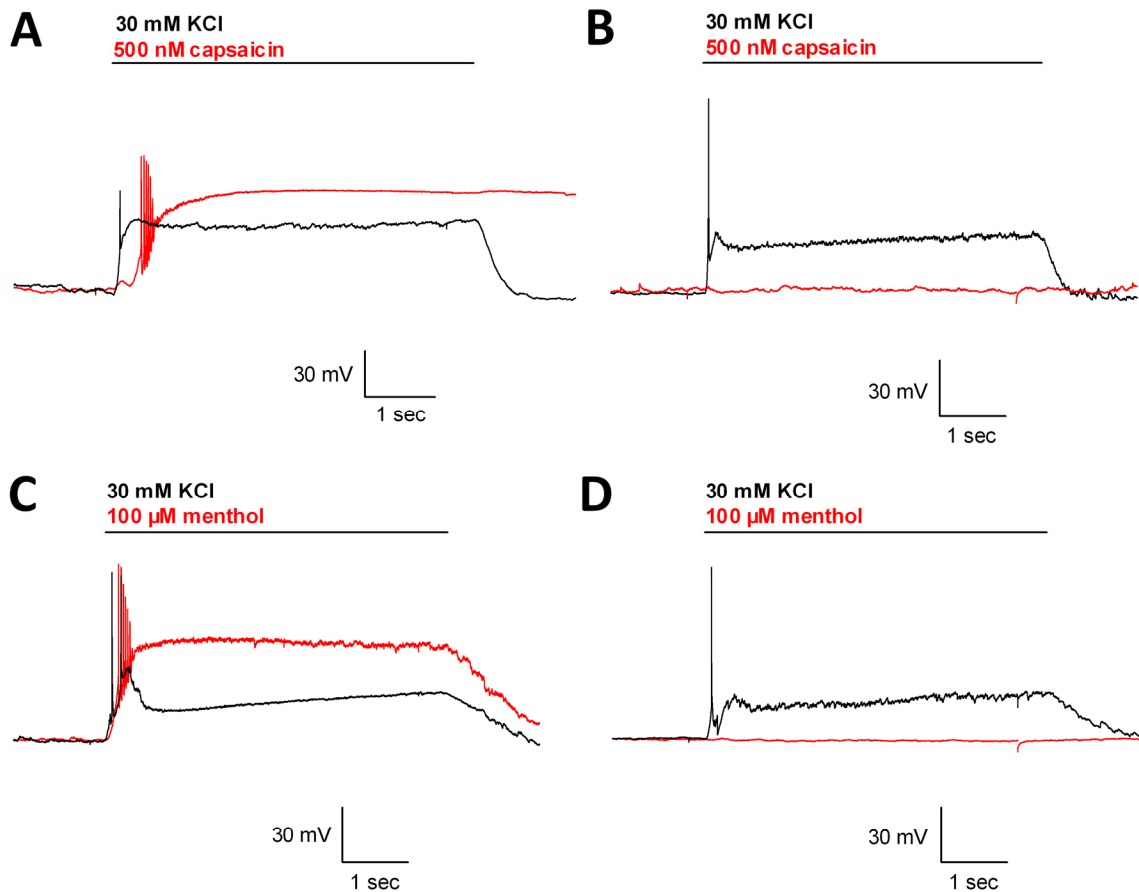


Figure S1. Agonist application to transfected neurons causes action potentials and strong depolarization. **A.** Example of a TRPV1-transfected neuron exposed to 5 s acute 30 mM KCl (black trace) or 500 nM capsaicin (red trace). Neurons were recorded in whole-cell current-clamp after adjusting the baseline membrane potential to -65 mV with small bias current when necessary. Although capsaicin-induced voltage changes were slow to return to baseline, they did so within ~20 s. **B.** The same as A except recording from a non-transfected neuron in the same culture. **C.** Example of a TRPM8-transfected neuron exposed to 5 s acute 30 mM KCl (black trace) or 100 μ M menthol (red trace). Neurons were recorded in current clamp after adjusting the baseline membrane potential to -65 mV as described in A. **D.** The same as C except recording from a non-transfected neuron in the same culture.

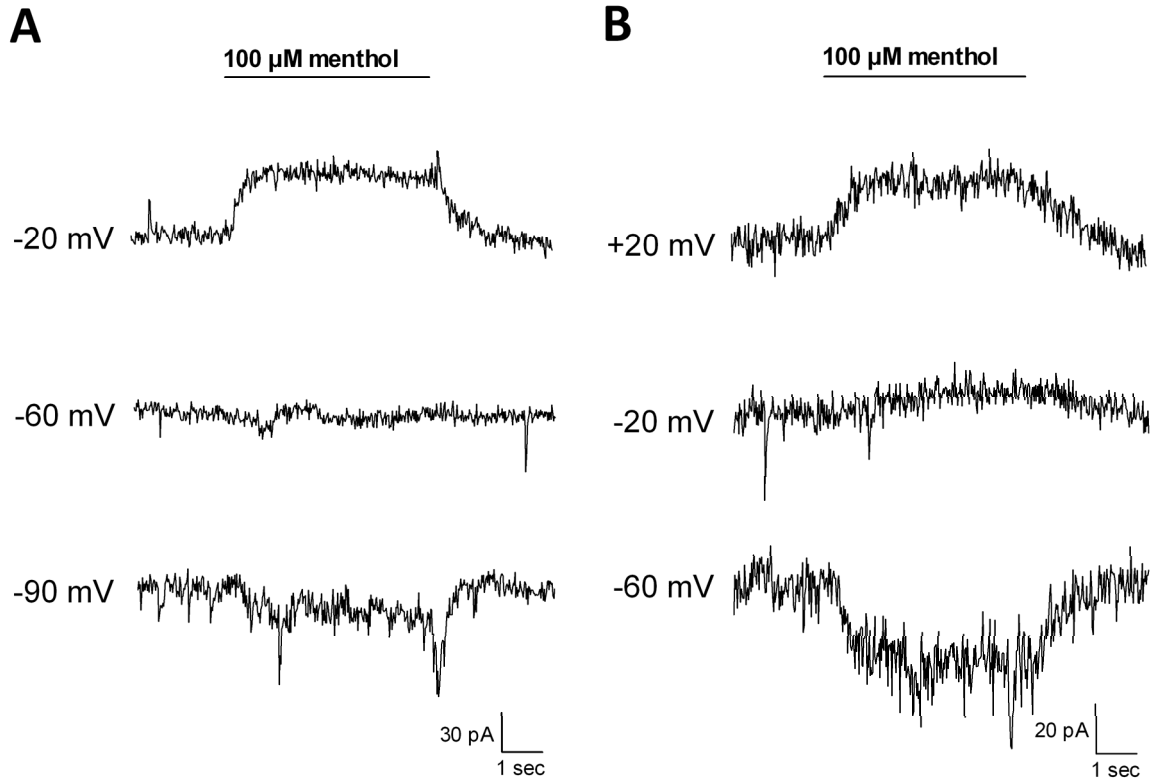


Figure S2. Menthol application to non-transfected neurons induces a current that changes direction with the chloride gradient. **A.** Example of a non-transfected neuron recorded in whole-cell voltage-clamp in the presence of 0.5 μM tetrodotoxin (TTX) with a cesium methanesulfonate internal pipette solution held at various membrane potentials during a local 5 s 100 μM menthol application. Note the change in current direction with potentials above and below ~ -60 mV. **B.** Example of a non-transfected neuron recorded in the presence of 0.5 μM TTX with a cesium chloride internal pipette solution held at various membrane potentials during a local 5 s 100 μM menthol application. Note the change in current direction with potentials above and below ~ -20 mV. These data are consistent with menthol gating a small GABA_A receptor-mediated current in non-transfected hippocampal neurons (see Fig 3G).

Chapter 3

Calcium-independent inhibitory G-protein signaling induces persistent presynaptic muting of hippocampal synapses

This chapter contains a previously published manuscript:

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Author contributions for the citation above:

D.C.C. designed and performed experiments, analyzed data, and wrote the paper. C.Y.C. performed electrophysiological experiments for Figure 2. K.L.H. performed calcium imaging experiments for Figure 1. S.M. designed experiments, performed experiments for Figure 2, analyzed data, and wrote the paper.

Abstract

Adaptive forms of synaptic plasticity that reduce excitatory synaptic transmission in response to prolonged increases in neuronal activity may prevent runaway positive feedback in neuronal circuits. In hippocampal neurons, for example, glutamatergic presynaptic terminals are selectively silenced, creating “mute” synapses, after periods of increased neuronal activity or sustained depolarization. Previous work suggests that cAMP-dependent and proteasome-dependent mechanisms participate in silencing induction by depolarization, but upstream activators are unknown. We, therefore, tested the role of calcium and G-protein signaling in silencing induction in cultured hippocampal neurons. We found that silencing induction by depolarization was not dependent on rises in intracellular calcium, either from extracellular or intracellular sources. Silencing was, however, pertussis toxin-sensitive, which suggests that inhibitory G-proteins are recruited. Surprisingly, blocking four common inhibitory G-protein-coupled receptors (GPCRs; adenosine A1 receptors, GABA_B receptors, metabotropic glutamate receptors, and CB1 cannabinoid receptors) and one ionotropic receptor with metabotropic properties (kainate receptors) failed to prevent depolarization-induced silencing. Activating a subset of these GPCRs (A1 and GABA_B) with agonist application induced silencing, however, which supports the hypothesis that G-protein activation is a critical step in silencing. Overall our results suggest that depolarization activates silencing through an atypical GPCR or through receptor-independent G-protein activation. GPCR agonist-induced silencing exhibited dependence on the ubiquitin-proteasome system, as was previously shown for depolarization-induced silencing, implicating the degradation of vital synaptic proteins in silencing by GPCR activation.

These data suggest that presynaptic muting in hippocampal neurons employs a G-protein-dependent but calcium-independent mechanism to depress presynaptic vesicle release.

Introduction

Adaptive forms of plasticity, including homeostatic synaptic plasticity, help maintain neuronal firing rates and prevent over-excitation, excitotoxicity, and information degradation caused by positive feedback inherent in glutamate signaling (Turrigiano, 1999; Turrigiano and Nelson, 2004; Maffei and Fontanini, 2009; Pozo and Goda, 2010). We have described adaptive presynaptic silencing after increased neuronal activity (Moulder et al., 2004; Moulder et al., 2006). Indeed, presynaptically silent (mute) synapses have been observed in several preparations, but induction and expression mechanisms remain unclear (Malenka and Nicoll, 1997; Voronin and Cherubini, 2004; Atasoy and Kavalali, 2006).

In hippocampal neurons, depolarization-induced presynaptic silencing is induced rapidly by strong depolarization akin to that generated by stroke, seizure, and spreading depression (Gido et al., 1997; Walz, 2000; Somjen, 2001; Moulder et al., 2004). Physiological action potential firing induces silencing over more protracted periods (Moulder et al., 2006), so presynaptic muting operates over a range of physiological, and perhaps pathophysiological, conditions. Adaptive presynaptic silencing is selective for glutamatergic terminals and is slowly reversible (Moulder et al., 2004). Expression involves altered vesicle priming and requires the ubiquitin-proteasome system (Moulder et al., 2006; Jiang et al., 2010), but upstream induction mechanisms remain uncertain.

Circumstantial evidence implicates calcium and cyclic adenosine monophosphate (cAMP) alterations in silencing induction. Calcium is involved in many forms of synaptic plasticity (Malenka, 1994; Fisher et al., 1997; Turrigiano, 2008). Prolonged decreases in cAMP signaling produce presynaptic silencing, and cAMP increases occlude

depolarization-induced silencing (Moulder et al., 2008). Furthermore, calcium-sensitive adenylyl cyclase activity, which increases cAMP levels, is important for normal recovery from silencing (Moulder et al., 2008). If decreased cAMP signaling is needed for depolarization-induced presynaptic silencing, we might expect involvement of inhibitory G-proteins in its induction. Despite this evidence, double knockout mice of the two major calcium-sensitive isoforms of adenylyl cyclase exhibit intact depolarization-induced silencing (Moulder et al., 2008), thereby questioning a direct role for either calcium or G-protein signaling. Clarification of how adaptive presynaptic silencing is induced will be crucial in identifying potential targets for therapies aimed at excitotoxicity-related dysfunction.

Here we explored whether calcium and inhibitory G-protein signaling are necessary for hippocampal adaptive presynaptic silencing. Surprisingly, intracellular calcium rises are unnecessary. However, we found that pertussis toxin, which inhibits $G_{i/o}$ signaling, reduced depolarization-induced silencing. Direct agonist activation of two classes of G-protein-coupled receptors (GPCRs) induced silencing, supporting the hypothesis that silencing is G-protein-dependent. In contrast, pharmacological blockade of five candidate receptors, including the two that induced silencing, failed to block depolarization-induced silencing. This suggests that depolarization activates an uncommon GPCR or a pertussis toxin-sensitive G-protein through a receptor-independent mechanism. Nevertheless, depolarization- and GPCR-induced muting share downstream mechanisms because both are sensitive to proteasome inhibition. Thus, adaptive presynaptic silencing is a unique form of calcium-independent, G-protein-dependent synaptic plasticity. Furthermore, we show that prolonged G-protein activation leads to

persistent presynaptic silencing, an effect distinct from classical acute presynaptic GPCR effects on vesicle release probability.

Materials and Methods

Cell culture:

Cultures of primary hippocampal neurons were prepared as previously described (Mennerick et al., 1995). Briefly, hippocampi were removed from postnatal 0-3 d male and female Sprague Dawley rat pups and enzymatically treated with papain (1 mg/ml) before mechanical dissociation. Cells were then plated in 35 mm culture dishes pre-coated with 0.15% agarose as either mass cultures, seeded at ~ 650 cells/mm² on a confluent layer of type I collagen (0.5 mg/ml), or as microisland cultures, seeded at ~ 100 cells/mm² on microdots of collagen. Plating medium contained Eagle's medium (Invitrogen) supplemented with 5% heat-inactivated horse serum, 5% fetal bovine serum, 17 mM D-glucose, 400 μ M glutamine, 50 U/ml penicillin, and 50 μ g/ml streptomycin. Cultures were maintained at 37°C in a humidified incubator under controlled atmospheric conditions (5% CO₂/95% air). Cytosine arabinoside (6.7 μ M) was added 3-4 d after plating to inhibit cell division. One half of the culture media was exchanged with Neurobasal medium (Invitrogen) plus B27 supplement 4-5 d after plating. All experiments were performed 10-14 d *in vitro*, and controls consisted of sibling cultures from the same litter that were treated and/or recorded on the same day to control for synapse development and culture conditions that vary by age and plating conditions. All 4 hr treatments were performed in the presence of ionotropic glutamate receptor blockers D-(-)-2-amino-5-phosphonopentanoic acid (D-APV; 50 μ M; Tocris) and 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX; 1 μ M; Tocris), and non-depolarized controls always consisted of equimolar NaCl addition to the media to

control for osmotic changes. Controls for calcium-free Neurobasal medium (Washington University Medical School Tissue Culture Support Center) supplemented with B27 and ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA) were given the identical medium except with 2 mM calcium and without EGTA.

Electrophysiology:

All whole-cell voltage-clamp recordings were performed on autaptic neurons in microisland culture. To control for variability in maturity and development, all experiments utilized similar numbers of neurons from sibling cultures on any given day. Data were collected with an Axopatch 200B or Multiclamp 700B amplifier interfaced to a Digidata 1322A or 1440A data acquisition board (Molecular Devices).

Before recording, the culture medium was exchanged for saline solution containing 138 mM NaCl, 4 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, 10 mM HEPES, and 25 μM D-APV with a pH of 7.25. For most experiments measuring excitatory postsynaptic currents (EPSCs), internal pipette solution consisted of 140 mM K-gluconate, 4 mM NaCl, 0.5 mM CaCl₂, 5 mM EGTA, and 10 mM HEPES at a pH of 7.25. For experiments measuring both EPSCs and inhibitory postsynaptic currents (IPSCs), 140 mM KCl was substituted for K-gluconate to provide a similar PSC driving force at the holding potential of -70 mV.

Electrode pipettes pulled from borosilicate glass (World Precision Instruments) had resistances of 2.5-6 MΩ, and access resistance was compensated 85-100%. Signals were sampled at 5-10 kHz and low-pass filtered at 2-5 kHz. Recordings were performed at room temperature, and membrane potential was typically clamped at -70 mV. Neurons

were recorded within 1 hr of exchanging the culture medium with the saline solution.

PSCs were evoked by a brief (1.5 ms) depolarization to 0 mV. Paired-pulse responses were evoked by 2 such depolarizations with an inter-stimulus interval of 50 ms. Sucrose-evoked EPSCs were elicited by 3 s local application of 0.5 M sucrose. All local solution applications utilized a multi-barrel perfusion system with the common perfusion port placed within 0.5 mm of the neuron under study and yielding solution exchange times of ~100 ms.

Calcium imaging:

All calcium imaging experiments utilized mass cultures. For fura-2 experiments, neurons were plated on glass-bottom dishes (MatTek Corporation). Cells were loaded with fura-2 by 60 min incubation with 5 μ M fura-2-acetoxymethyl ester (Invitrogen) and 0.1% Pluronic F-127 (Invitrogen) in Neurobasal medium (pH of 7.2) at room temperature, washed with Neurobasal medium and incubated for another 60 min to allow for ester hydrolysis. After loading, the cells were imaged on an Eclipse TE300 inverted microscope using a 40x (1.3 numerical aperture) oil immersion objective (Nikon). The microscope was equipped with a 75 W xenon arc lamp and a cooled CCD camera (Cooke Corp.). The fluorescence excitation was provided by a band-specific filter (340 and 380 nm; Semrock) in combination with DM400 dichroic beam splitter (Nikon). Pairs of images were collected at alternate excitation wavelengths. The images were divided by one another to yield ratio values for individual cell bodies after subtracting the matching background. Imaging was performed at room temperature, but cultures were returned to 37°C for the period between images early in the treatment and images at the end of the 4

hr treatment. For this reason, the same neurons were represented at baseline and 5 min time points, but separate fields of neurons were used for 4 hr time points in Figure 1.

MetaFluor software (Molecular Devices) was used for image acquisition and analysis.

Fluo-4 was loaded into cells by addition of Fluo-4 AM (2 μ M; Invitrogen) to the culture media 30 min prior to the end of the 4 hr KCl treatment. Medium was then exchanged for extracellular recording saline containing elevated KCl (34 mM total) and 0 mM CaCl₂. Cells were imaged at room temperature using an Eclipse TE2000-S inverted microscope with 40x (0.6 numerical aperture) objective (Nikon) and a cooled 12-bit CCD camera (Photometrics). Epifluorescence was provided by a metal halide lamp, and images were acquired at 1 Hz using MetaMorph software (Molecular Devices). Calcium was briefly elevated to 0.5 mM for 5-10 s after 5 s of baseline in 0 mM calcium.

FM1-43 loading and immunocytochemistry:

All FM1-43 imaging experiments utilized mass cultures plated on glass coverslips. FM1-43 loading and vesicular glutamate transporter 1 (vGluT-1) immunolabeling experiments were performed as previously described (Moulder et al., 2006; Moulder et al., 2010). Briefly, within 60 s of replacing media with fresh saline, active presynaptic terminals were labeled for 2 min with 10 μ M fixable FM1-43 (FM1-43FX; Invitrogen) in recording saline supplemented with 45 mM KCl and 1 μ M NBQX. Cultures were immediately washed for 5 s with extracellular recording saline containing 500 μ M Advasep-7 (CyDex) and 1 μ M NBQX followed by saline plus 1 μ M NBQX for 10 min. Afterwards, cells were fixed for 10 min in 4% paraformaldehyde/0.2% glutaraldehyde in phosphate buffered saline (PBS), washed with PBS, and incubated in a

blocking solution (2% normal goat serum/0.08% Triton X-100) for 15 min before 3 hr incubation in guinea pig anti-vGluT-1 antibody (Millipore) diluted 1:2000 in blocking solution. After washing with PBS, cells were incubated for 40 min in Alexa Fluor 647-conjugated anti-guinea pig antibody (Invitrogen) diluted 1:500 in PBS. After washing again with PBS, coverslips were removed from culture dishes and mounted onto glass microscope slides with Fluoromount-G (Southern Biotechnology).

Confocal images were acquired at random locations throughout the dish by an observer naïve to the experimental conditions using a C1 scanning confocal laser attached to an inverted Eclipse TE300 microscope with a 60x objective (1.4 numerical aperture) and EZ-C1 software (Nikon). Alternating 543 nm and 633 nm laser lines were used to obtain z-stack images while pixel dwell time, image resolution, gain settings, field of view size, and z-stack parameters were held constant throughout all conditions from a single experiment. Monochrome images were converted into two-dimensional projected images and analyzed using MetaMorph software.

Trypan blue staining:

For experiments assessing toxicity via trypan blue staining, mass cultures were treated for 5 min with 0.4% trypan blue at 37°C in a humidified incubator under controlled atmospheric conditions (5% CO₂/95% air). After one PBS wash at room temperature, neurons were fixed for 10 min in 4% paraformaldehyde/0.2% glutaraldehyde in PBS. After 3 additional PBS washes, 10 random fields of cells were counted in each dish using a 20x objective. Trypan-positive nuclei were counted under bright field and identified as unhealthy neurons, and phase-bright cell bodies devoid of

trypan staining were counted under phase contrast and identified as healthy neurons.

Data analysis:

Electrophysiology data were collected and analyzed with pClamp 9 software (Molecular Devices). For electrically-evoked PSCs, leak was subtracted offline before measuring peak amplitude, and the amplitudes of at least three responses were averaged for each cell. For sucrose-evoked EPSCs, currents were integrated over the rise and decay of the response to 10% of the steady-state current. This provided an estimate of the postsynaptic charge transfer corresponding to the readily releasable vesicle pool.

In calcium-imaging experiments using fura-2, the ratio values were normalized to the baseline values (in which no treatment had been applied) to control for differences in baseline calcium concentration. Normalization was achieved by dividing by the first value for each cell under baseline conditions, and 4 hr time points were normalized to the average of the ratios at baseline. Values from at least 5 images were averaged for each neuron at each time point. To calculate the change in fluorescence in fluo-4 experiments, somatic fluorescence intensities from three images were averaged at baseline and subtracted from the average intensity of three images near the peak of fluorescence. The change in fluorescence was normalized to the average baseline fluorescence value to account for variability in fluo-4 loading.

To determine the percentage of active glutamatergic synapses, regions were manually drawn around 10 vGluT-1 puncta each from 5 images (fields) for each coverslip in a single experiment by an observer naïve to experimental conditions. The strategy of using 5 fields per condition equalized the contribution of a single condition to

the final results. Regions were drawn to include the pixels above threshold in the vGluT-1-positive punctum but not larger than the punctum in order to minimize the contribution of background pixels and/or neighboring puncta to the analysis. Thresholds were applied to all FM1-43FX images before vGluT-1 regions were loaded into the FM1-43FX image. “Active” synapses were defined as those regions that reached at least 10 pixels above threshold in the FM1-43FX image (Moulder et al., 2006). Cluster correlation analyses (Galbraith et al., 2010) and ANOVA statistics applied to the first FM1-43FX/vGluT-1 correspondence experiment (Fig. 1D) found no significant correlations between fields within an experiment (data not shown), so we treated the percentage of active synapses from each field as statistically independent samples throughout the manuscript.

For trypan blue toxicity experiments, the total numbers of healthy and unhealthy neurons were added together from all 10 fields in each dish. The percentage of healthy neurons was then calculated as the number of healthy neurons divided by the total neurons counted (healthy and unhealthy) in each dish.

Graphs were created using SigmaPlot software (Systat Software Inc.). All data were presented as mean \pm SEM unless otherwise indicated. To reduce type 1 error that may result from non-significant cluster correlations (Galbraith et al., 2010), all experiments utilized yoked controls (e.g. sibling cultures; see methods above), and we analyzed data conservatively using unpaired statistics with additional corrections (i.e. for multiple comparisons) as appropriate. Unpaired Student's *t* test was used to determine significance for comparisons of two groups while Bonferroni correction for multiple comparisons was applied to analyses of experiments involving more than two conditions. A *p* value of < 0.05 was required for significance.

Materials:

SCH50911, bicuculline, LY341495, (1S,3R)-ACPD (ACPD), and 2-chloro-N⁶-cyclopentyladenosine (CCPA) were purchased from Tocris. BAPTA-AM (acetoxymethyl ester derivative of bis(2-aminophenoxy)ethane tetraacetic acid) was purchased from Invitrogen while 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) was purchased from Research Biochemicals Inc. Carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG-132) was purchased from Enzo Life Sciences. All other chemicals and reagents were purchased from Sigma unless otherwise stated.

Results

Depolarization silences presynaptic terminals in the absence of extracellular calcium:

Calcium is necessary for multiple forms of hippocampal synaptic plasticity, so we determined the role of calcium influx in adaptive presynaptic silencing by removing calcium from the extracellular media during a depolarizing induction challenge known to produce strong presynaptic muting. We used an induction paradigm of 30 mM KCl for 4 hr in the presence of ionotropic glutamate receptor blockers D-APV (50 μ M) and NBQX (1 μ M). The short, strong induction circumvents inevitable developmental confounds of longer interventions with calcium and G-protein signaling and mimics pathophysiological extracellular potassium concentrations during stroke or seizure (Gido et al., 1997; Walz, 2000). 30 mM KCl clamps the membrane potential at -20 to -30 mV (Moulder et al., 2003; Crawford et al., 2009) and induces adaptive presynaptic silencing without detectable changes in synaptic structure, vesicle release probability (p_r), or postsynaptic function (Moulder et al., 2004; Moulder et al., 2006). We incubated mass cultures in either calcium-rich culture media or calcium-free culture media supplemented with 500 μ M EGTA to buffer residual free calcium. Using the ratiometric calcium indicator fura-2, we verified that calcium-free media prevented rises in cytosolic free calcium concentration normally produced by KCl depolarization (Fig. 1A, B). Control neurons also exhibited a late rise in cytosolic free calcium concentration (Fig. 1B). We speculate that temperature and pH variations between imaging sessions (at room temperature) and intervening periods (at 37°C) may have contributed to cellular stress and calcium influx in non-depolarized neurons. Regardless, calcium-free media prevented the rise in

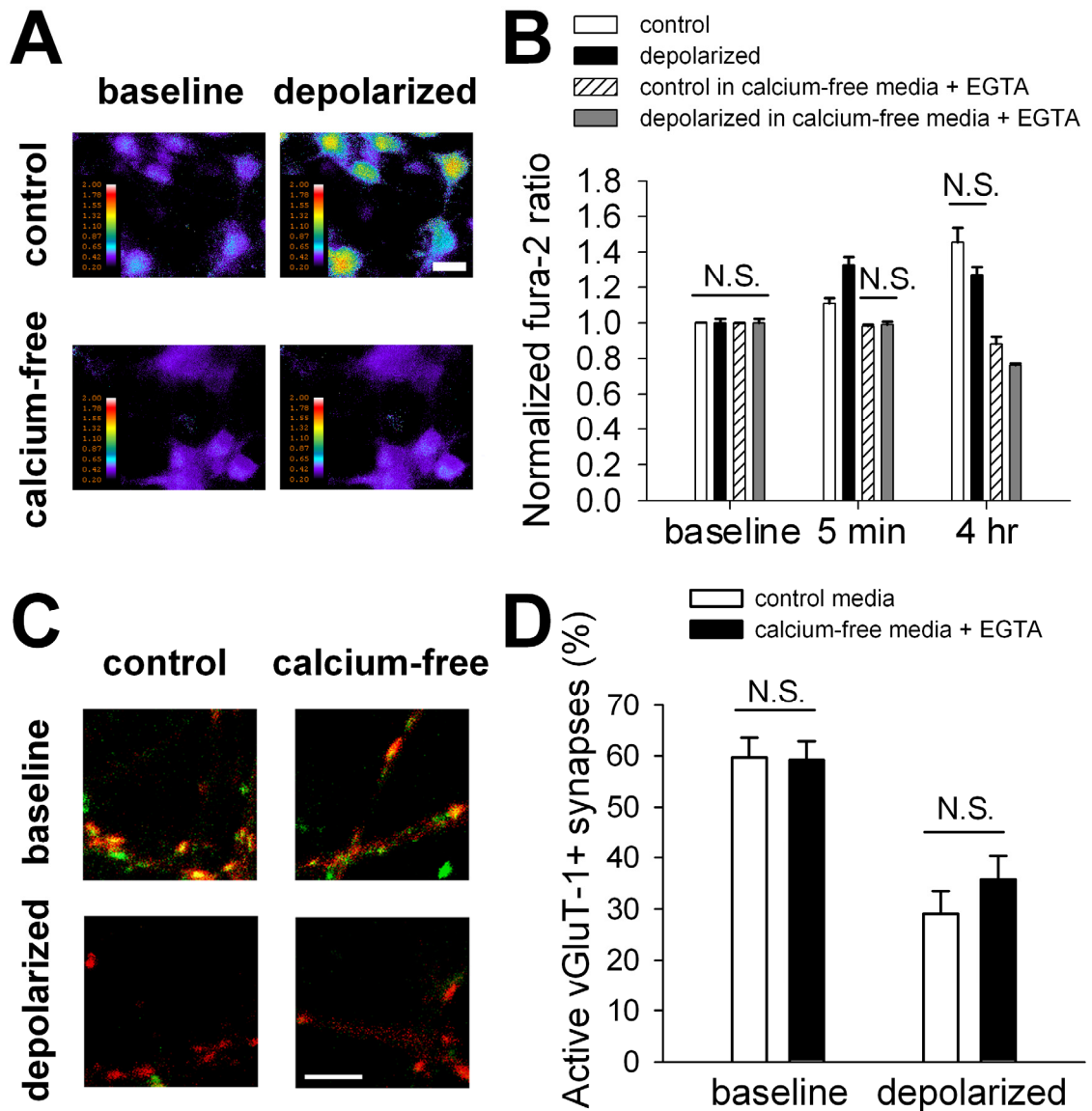


Figure 1. Depolarization-induced presynaptic silencing is not dependent on calcium influx. **A.** Pseudo-colored images of the fura-2 ratio (indicating calcium concentration) in neuronal cell bodies before (baseline) and < 1 min after (depolarized) application of 30 mM KCl. Neurons were switched from calcium-containing media to either calcium-containing (control) media or to calcium-free media supplemented with 500 μ M EGTA when KCl depolarization began. Scale bar denotes 25 μ m. **B.** Summary of fura-2 ratio normalized to baseline values in neurons similar to **A** at two time points following addition of either 30 mM NaCl (control) or 30 mM KCl (depolarized) to calcium-containing media or calcium-free media supplemented with 500 μ M EGTA. All comparisons within a single time point were significantly different ($p < 0.05$ with Bonferroni correction for multiple comparisons) except those indicated with “N.S.” meaning “not significant” ($n = 17-62$ neurons). Thus, the calcium-free media with EGTA effectively prevented cytosolic free calcium level rises in both control and depolarized neurons. **C.** Vesicular glutamate transporter 1 (vGluT-1) immunoreactivity to identify

glutamatergic presynaptic terminals (red) with superimposed FM1-43FX labeling of active presynaptic terminals (green). Neurons were treated 4 hr with either 30 mM NaCl (baseline) or 30 mM KCl (depolarized) in either calcium-containing media (control) or calcium-free media supplemented with 500 μ M EGTA (calcium-free). Scale bar denote 5 μ m. **D.** Summary of the percentage of active glutamatergic presynaptic terminals based on vGluT-1/FM1-43FX correspondence as shown in C. All comparisons were significantly different ($p < 0.05$ with Bonferroni correction for multiple comparisons) except those indicated with “N.S.” ($n = 20$ fields of 10 vGluT-1 puncta each from 4 independent experiments).

cytosolic free calcium concentration in both control and depolarized neurons over 4 hr (Fig. 1B). We conclude, therefore, that our calcium-free media supplemented with EGTA effectively prevented calcium influx.

To assess presynaptic silencing after depolarization in the absence of calcium influx, we performed FM1-43FX labeling in the presence of calcium followed by retrospective immunostaining for vesicular glutamate transporter 1 (vGluT-1) to identify active glutamatergic terminals. In agreement with previous experiments under normal calcium conditions (Moulder et al., 2004), neurons depolarized in calcium-free media plus EGTA exhibited a decrease in the percentage of vGluT-1 terminals that co-localize with FM1-43FX (Fig. 1C, D). This suggests that calcium influx is not necessary to induce adaptive presynaptic silencing.

Depolarization silences presynaptic terminals in the absence of intracellular free calcium:

Although influx of extracellular calcium is not necessary for induction of adaptive presynaptic silencing, calcium rises from intracellular sources may participate. To prevent intracellular calcium rises, we loaded cells with the fast intracellular calcium chelator BAPTA using the cell-permeant precursor BAPTA-AM. Because normal action potential-driven neurotransmitter release is dependent on fast rises in local presynaptic calcium concentration and would be depressed by BAPTA-AM incubation, we used calcium-independent hypertonic sucrose-evoked excitatory postsynaptic currents (EPSCs) to determine the size of the readily-releasable pool of vesicles (Rosenmund and Stevens, 1996), which is proportional to the total number of active synapses (Moulder et al., 2004). For these experiments, we used autaptic neurons in microisland cultures and

applied hypertonic sucrose to evoke EPSCs during whole-cell recordings. Autaptic neurons were used because their isolation eliminates confounding polysynaptic effects during electrophysiological measurements. Similar to previous results (Rosenmund and Stevens, 1996), sucrose-evoked EPSCs were unaffected by 4 hr incubation with 20 μ M BAPTA-AM in calcium-free media (Fig. 2A, B). In contrast electrically-evoked EPSCs, which are dependent on presynaptic calcium influx, were strongly depressed after 4 hr BAPTA chelation (Fig. 2A, B). These results suggest that 4 hr BAPTA incubation strongly chelates intracellular free calcium.

To test if intracellular rises in calcium concentration are necessary for presynaptic silencing, we depolarized neurons for 4 hr in 30 mM KCl in the presence of 2 mM calcium or in calcium-free media containing 20 μ M BAPTA-AM. To ensure that intracellular calcium rises during induction did not saturate the ability of BAPTA to chelate calcium, we performed calcium imaging of treated neurons using the calcium indicator fluo-4. Briefly raising extracellular calcium from 0 mM to 0.5 mM increased cytosolic free calcium concentration in control neurons after 4 hr depolarization in calcium-rich media, as assessed by changes in somatic fluo-4 fluorescence ($45.5 \pm 4.5\%$ increase; $n = 40$; Fig. 2C). This rise in cytosolic free calcium was not observed in neurons after incubation in calcium-free media supplemented with BAPTA-AM ($1.0 \pm 1.2\%$ increase; $n = 40$; $p = 1.0 \times 10^{-15}$; Fig. 2C). BAPTA, therefore, effectively chelates intracellular free calcium, even following a sustained depolarization.

To test whether BAPTA-AM incubation blocks presynaptic silencing, we incubated in BAPTA-AM and compared neurons after the KCl challenge to neurons challenged with an equimolar NaCl control. As shown previously, the 4 hr depolarizing

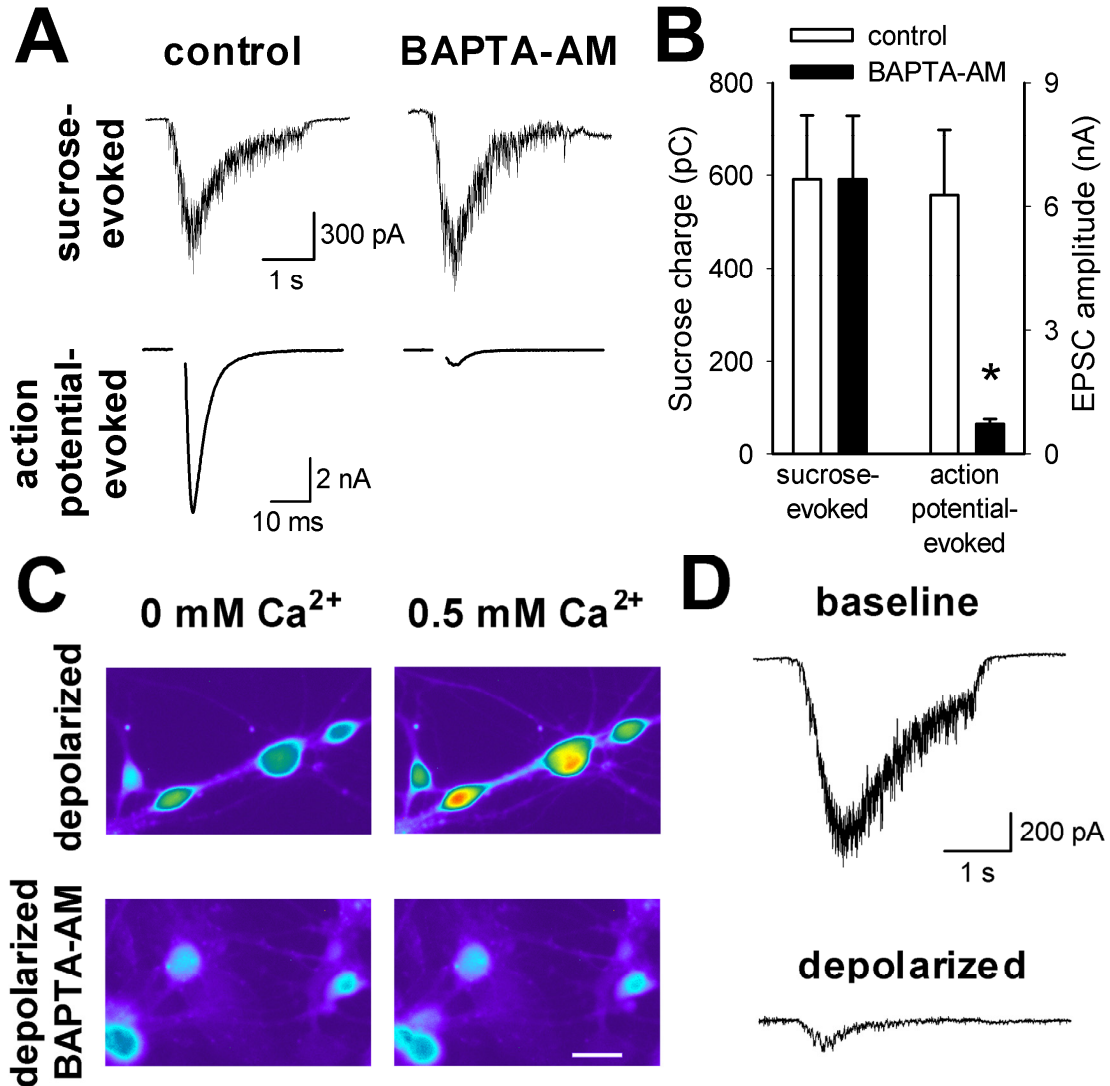


Figure 2. Depolarization-induced presynaptic silencing is not dependent on increases in intracellular free calcium from any source. **A.** Representative examples of sucrose-evoked excitatory postsynaptic currents (EPSCs) and action potential-evoked EPSCs elicited in autaptic neurons after 4 hr treatment in normal calcium-containing media (control) or calcium-free media supplemented with 20 μ M of the cell-permeant fast calcium chelator BAPTA-AM. **B.** Summary of results for sucrose-evoked EPSC charge and action potential-evoked EPSC amplitude after treatment with control or with calcium-free, BAPTA-AM-supplemented extracellular media in sister cultures ($n = 9-11$ neurons). $*p < 0.05$. **C.** Example fields of neurons filled with the calcium indicator fluo-4 before (0 mM) and after (0.5 mM) perfusion of CaCl₂. Neurons were pre-treated for 4 hr with 30 mM KCl in either calcium-containing media (depolarized) or calcium-free media supplemented with 20 μ M BAPTA-AM (depolarized BAPTA-AM). Scale bar denotes 50 μ m. **D.** Representative examples of sucrose-evoked EPSCs in autaptic neurons after 4 hr treatment in calcium-free media supplemented with 20 μ M BAPTA-AM and either 30 mM NaCl (baseline) or 30 mM KCl (depolarized).

challenge in calcium-rich media depressed sucrose-evoked EPSCs by ~45% (control: 1224.9 ± 147.7 pC; depolarized: 670.1 ± 135.7 pC; $n = 29-30$ neurons; $p < 0.01$; see also Moulder et al., 2004). Despite BAPTA-AM incubation in calcium-free media, the charge of sucrose-evoked EPSCs was still significantly depressed in KCl-challenged neurons compared with sibling controls (control: 581 ± 100 pC; depolarized: 168 ± 27 pC; $n = 26-29$; $p < 0.0001$; Fig. 2D). These results argue strongly that adaptive presynaptic silencing is calcium-independent.

Activation of inhibitory G-proteins is necessary for depolarization-induced presynaptic silencing:

Indirect evidence has previously implicated decreased cAMP levels in depolarization-induced presynaptic silencing (Moulder et al., 2008). Thus, prolonged activation of inhibitory G-proteins, which inhibit adenylyl cyclase activity and cAMP production, could participate in adaptive muting. We, therefore, tested the hypothesis that inhibitory G-proteins are necessary for depolarization-induced silencing.

We pre-incubated neurons for 24 hr in 500 ng/ml pertussis toxin, which, by adenosine diphosphate-ribosylation of the $\alpha_{i/o}$ subunit, prevents inhibitory G-protein activation. This interferes with α subunit signaling and blocks the association of $G\alpha_{i/o}$ subunits with their upstream GPCRs (Brown and Sihra, 2008). To confirm that pertussis toxin treatment itself did not induce toxicity, we used trypan blue staining to identify unhealthy neurons. The percentage of healthy neurons did not differ between control neurons and those treated 24 hr with 500 ng/ml pertussis toxin (control: $92.2 \pm 1.6\%$; pertussis toxin: $93.5 \pm 1.0\%$; $n = 4$ dishes; $p = 0.54$). After 24 hr in pertussis toxin, we

co-incubated with 30 mM KCl or an equimolar control during an additional 4 hr in pertussis toxin. After treatment, we assessed synaptic function by measuring EPSC amplitudes in autaptic neurons and vGluT1/FM1-43FX correspondence in mass cultures. Pertussis toxin had no effect on EPSC amplitude in non-depolarized neurons, confirming that the toxin did not compromise cell health, but it prevented depolarization-induced depression of EPSC amplitude (Fig. 3A, B). Pertussis toxin did not, however, alter paired-pulse depression in any of the experimental groups (control: $29.9 \pm 5.6\%$; pertussis toxin: $18.0 \pm 4.3\%$; depolarized: $8.3 \pm 6.2\%$; depolarized + pertussis toxin: $20.7 \pm 5.9\%$; $n = 13-14$ neurons per condition; $p > 0.05$; also see supplemental Fig. 1A), as is expected from acute effects of GPCR activation on vesicle release probability (Mennerick and Zorumski, 1995; Dobrunz and Stevens, 1997; Zucker and Regehr, 2002; Brown and Sihra, 2008). Similarly pertussis toxin prevented the depolarization-induced decrease in active synapses measured via FM1-43FX/vGluT-1 correspondence (Fig. 3C, D). Together these results suggest that activation of inhibitory G-proteins is necessary for depolarization-induced presynaptic silencing.

A1 receptor activation is not required for depolarization-induced presynaptic silencing:

To explore the role of GPCRs upstream of inhibitory G-proteins in presynaptic silencing, we examined whether the G-protein-coupled A1 adenosine receptor is involved. The A1 receptor is an excellent candidate since increased adenosine levels in the extracellular space occur via calcium-independent mechanisms: conversion from adenosine triphosphate (Latini and Pedata, 2001), which is released from astrocytes (Wang et al., 2000), and release by transporters (Dunwiddie and Masino, 2001).

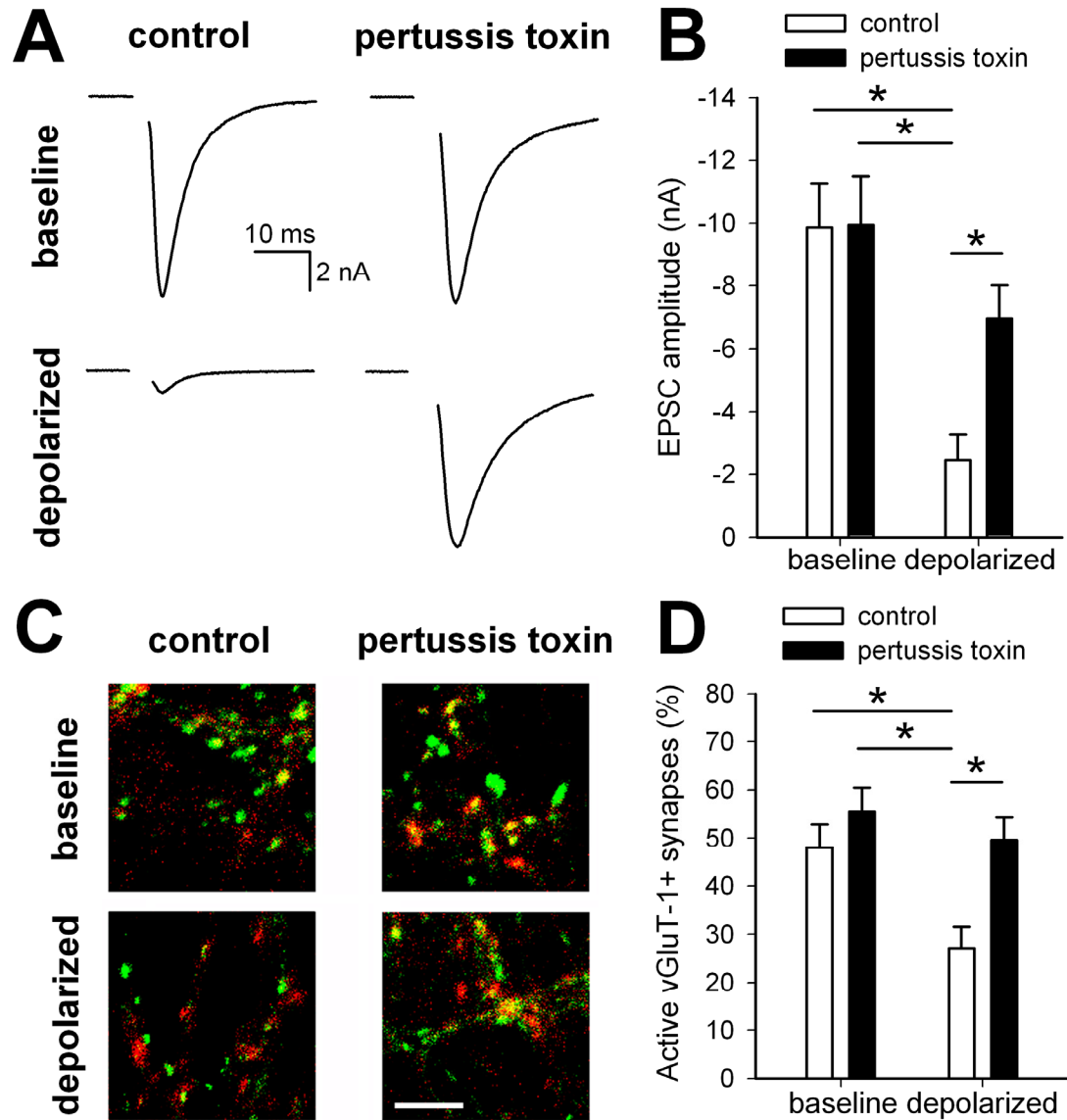


Figure 3. Activation of inhibitory G-proteins is necessary for depolarization-induced presynaptic silencing. **A.** Example action potential-evoked EPSCs from autaptic neurons treated 24 hr with or without 500 ng/ml pertussis toxin before 4 hr co-application with 30 mM NaCl (baseline) or 30 mM KCl (depolarized). **B.** Summary of EPSC amplitudes from autaptic neurons treated as in A ($n = 13-14$ neurons). $*p < 0.05$ with Bonferroni correction for multiple comparisons. **C.** Example images of vGluT-1 (red) and FM1-43FX (green) correspondence in neurons treated 24 hr with or without 500 ng/ml pertussis toxin before 4 hr baseline or depolarized. Scale bar denote 5 μm . **D.** Summary of conditions represented in C ($n = 25$ fields from 5 independent experiments). $*p < 0.05$ with Bonferroni correction for multiple comparisons.

Adenosine release has also been implicated in neuroprotection, in part through acute A1 receptor-mediated inhibition of presynaptic glutamate release (Stone et al., 2009).

Additionally, A1 receptors selectively inhibit synaptic transmission at excitatory, not inhibitory, terminals in the hippocampus (Yoon and Rothman, 1991), possibly explaining the selectivity of adaptive presynaptic silencing for excitatory over inhibitory terminals (Moulder et al., 2004).

To test whether A1 receptor activation is necessary for depolarization-induced presynaptic silencing, we incubated neurons with depolarizing KCl or equimolar NaCl control in the presence of the selective A1 antagonist 200 nM DPCPX. The decrease in EPSC amplitude in depolarized neurons treated with DPCPX was comparable to ESPC depression from neurons incubated in the absence of DPCPX (Fig. 4A, B). DPCPX incubation also did not alter paired-pulse depression (control: $11.8 \pm 4.8\%$; DPCPX: $16.4 \pm 5.1\%$; depolarized: $11.0 \pm 8.7\%$; depolarized + DPCPX: $7.3 \pm 11.7\%$; $n = 9$ neurons per condition; $p > 0.05$; also see supplemental Fig. 1B). Additionally, we assessed presynaptic silencing by measuring the percentage of active glutamatergic presynaptic terminals via FM1-43FX/vGluT-1 correspondence. In neurons treated 4 hr with 30 mM KCl, the percentage of vGluT-1-positive presynaptic terminals that co-localize with FM1-43FX fluorescence was decreased from control, regardless of whether the neurons had been co-incubated with DPCPX (Fig. 4C, D). We noticed a trend-level difference in the percentage of active synapses in depolarized neurons treated with DPCPX compared to depolarized neurons without DPCPX (depolarized: $10.6 \pm 1.5\%$; depolarized + DPCPX: $17.4 \pm 2.1\%$; $p = 0.052$ after correction for multiple comparisons), potentially suggesting a minor role of adenosine A1 receptors in depolarization-induced silencing.

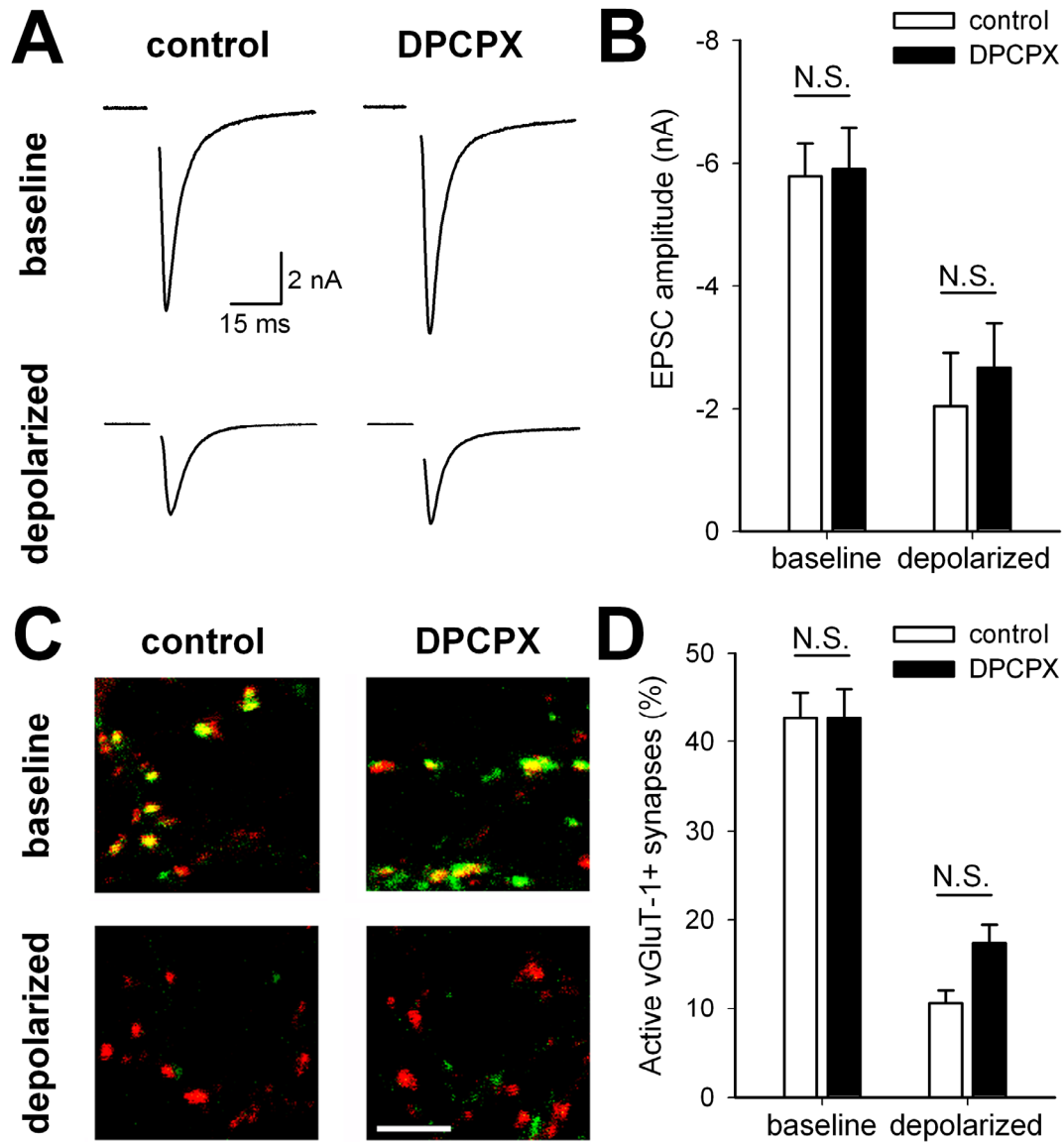


Figure 4. A1 adenosine receptor activation is not necessary for depolarization-induced presynaptic silencing. **A.** Example action potential-evoked EPSCs from autaptic neurons treated 4 hr with 30 mM NaCl (baseline) or 30 mM KCl (depolarized) in the presence or absence of 200 nM DPCPX, an A1 receptor antagonist. **B.** Summary of EPSC amplitudes from autaptic neurons treated as in **A** ($n = 9$ neurons). All comparisons were significantly different ($p < 0.05$ with Bonferroni correction for multiple comparisons) except those indicated (N.S.). **C.** Example images of vGluT-1 (red) and FM1-43FX (green) correspondence in neurons treated 4 hr under baseline or depolarized conditions in the presence or absence of 200 nM DPCPX. Scale bar denotes 5 μ m. **D.** Summary of conditions represented in **C** ($n = 65$ fields from 13 independent experiments). All comparisons were significantly different ($p < 0.05$ with Bonferroni correction for multiple comparisons) except those indicated (N.S.).

Despite this trend, however, there was still a significant decrease in the percentage of active synapses in the presence of DPCPX compared to control (DPCPX: $42.6 \pm 3.4\%$; depolarized + DPCPX: $17.4 \pm 2.1\%$; $p = 1.7 \times 10^{-8}$ after correction for multiple comparisons). Together these data suggest that blocking A1 receptor activation during depolarization does not prevent the induction of adaptive presynaptic silencing.

GABA_B, metabotropic glutamate, CB1, and kainate receptors are not required for depolarization-induced presynaptic silencing:

Although A1 adenosine receptor activation is not necessary to induce adaptive presynaptic silencing (Fig. 4), activation of inhibitory G-proteins is critical (Fig. 3). Multiple GPCRs linked to inhibitory G-proteins are expressed in rodent hippocampus. Our culture preparation, however, does not contain modulatory projection neurons from outside the hippocampus, so the list of GPCRs with ligands available *in vitro* is restricted. The calcium independence of induction also excludes transmitters/modulators released by classical calcium-dependent exocytosis. Nevertheless, reverse transport of γ -aminobutyric acid (GABA; Schwartz, 1982; Gaspary et al., 1998), reverse transport of glutamate (Nicholls and Attwell, 1990; Szatkowski et al., 1990), and non-classical cannabinoid signaling (Di Marzo and Deutsch, 1998; Di Marzo et al., 1998; Wilson and Nicoll, 2001) could elevate ambient modulator levels and induce silencing in response to depolarization.

We first tested whether the G-protein-coupled GABA_B receptor is responsible for inducing adaptive presynaptic silencing. To prevent GABA_B receptor activation, we incubated neurons in the presence of the GABA_B receptor antagonist SCH50911 (50

μM). To test effectiveness of SCH50911 in our system, we co-applied SCH50911 acutely to our autaptic neurons with the GABA_B receptor agonist baclofen (10 μM). Baclofen depressed evoked EPSCs and IPSCs by $65.4 \pm 8.1\%$, and addition of SCH50911 recovered the PSCs to $96.0 \pm 4.8\%$ of their original amplitude ($n = 10$ neurons; Fig. 5A). Incubation of mass cultures in SCH50911 for 4 hr significantly increased the percentage of active glutamatergic presynaptic terminals from baseline (Fig. 5B), suggesting a small endogenous GABA_B receptor tone in this data set. Despite this effect on baseline silent synapses, SCH50911 did not prevent the decrease in the percentage of active synapses induced by depolarization (Fig. 5B). These results demonstrate that, despite effectiveness of SCH50911, GABA_B receptor activation is not involved in depolarization-induced adaptive presynaptic silencing. Similar results were obtained with the GABA_A receptor antagonists bicuculline and picrotoxin (supplemental Fig. 2), confirming that GABA signaling is not involved in depolarization-induced presynaptic silencing.

Other plausible candidate GPCRs for the induction of adaptive presynaptic silencing include G_{i/o}-coupled metabotropic glutamate receptors (mGluRs). LY341495, a non-selective mGluR antagonist at concentrations above 22 μM (Schoepp et al., 1999), reversed acute EPSC depression by a near-saturating concentration of the mGluR agonist ACPD ($33.9 \pm 7.2\%$ depression in 50 μM ACPD; $92.2 \pm 6.8\%$ recovery in ACPD plus 25 μM LY341495; $n = 7-8$ neurons; Fig. 5C). However, LY341495 failed to alter baseline active glutamatergic synapses or presynaptic silencing in response to depolarization (Fig. 5D). These results indicate that antagonism of mGluR activation does not prevent depolarization-induced presynaptic silencing.

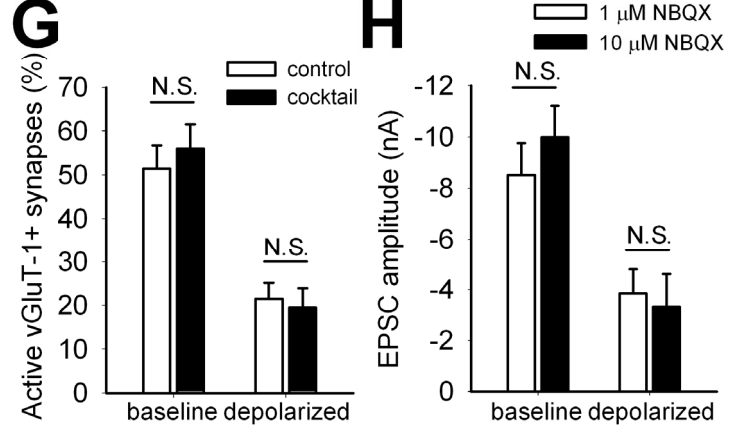
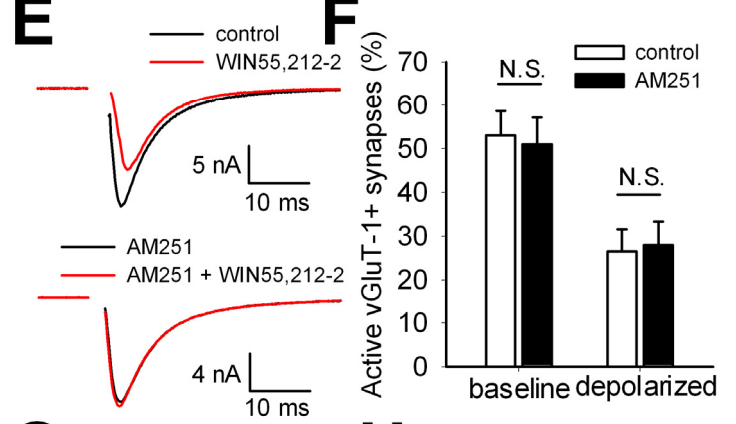
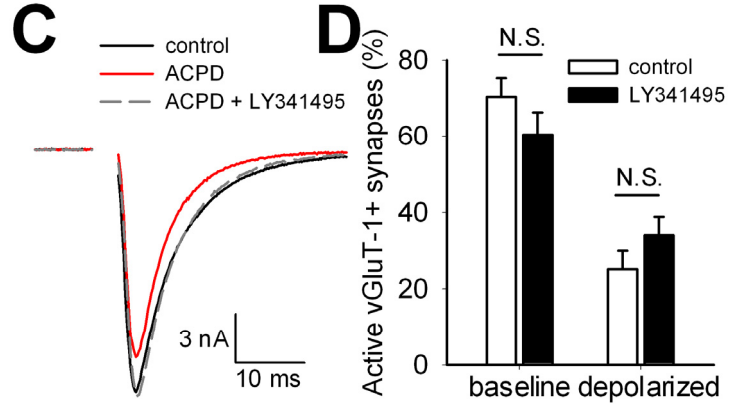
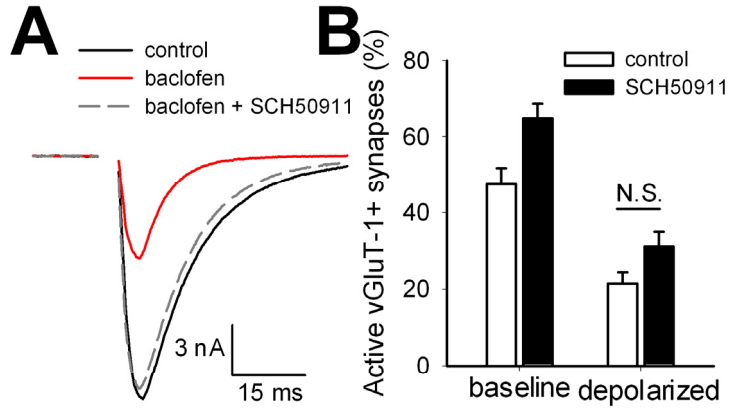


Figure 5. None of likely candidate GPCRs is necessary for depolarization-induced silencing. **A.** Example action potential-evoked IPSCs from a single autaptic neuron after acutely-applied extracellular saline (control), 10 μ M baclofen (a GABA_B receptor agonist), or 10 μ M baclofen plus 50 μ M SCH50911 (a GABA_B receptor antagonist). **B.** Summary of experiments measuring vGluT-1/FM1-43FX correspondence in neurons treated 4 hr with 30 mM NaCl (baseline) or 30 mM KCl (depolarized) in the presence or absence of 50 μ M SCH50911 ($n = 35$ fields from 7 independent experiments). All comparisons were significantly different ($p < 0.05$ with Bonferroni correction for multiple comparisons) except those indicated (N.S.). **C.** Example action potential-evoked EPSCs from a single autaptic neuron after acutely-applied control, 50 μ M ACPD (a mGluR agonist), or 50 μ M ACPD plus 25 μ M LY341495 (a mGluR antagonist). **D.** Summary of experiments measuring vGluT-1/FM1-43FX correspondence in neurons treated for 4 hr under baseline or depolarized conditions in the presence or absence of 25 μ M LY341495 ($n = 25$ fields from 5 independent experiments). All comparisons were significantly different ($p < 0.05$ with Bonferroni correction for multiple comparisons) except those indicated (N.S.). **E.** Example action potential-evoked EPSCs from two different autaptic neurons: one after acutely-applied control or 200 nM WIN55,212-2 (a CB1 agonist) and the other after 1-2 hr pre-treatment with 1 μ M AM251 (a CB1 antagonist), and continued AM251 application or AM251 plus WIN55,212-2. The AM251 pretreatment with between-cell comparison was used to mitigate against the very slow reversibility of WIN55,212-2. **F.** Summary of experiments measuring vGluT-1/FM1-43FX correspondence in neurons treated for 4 hr under baseline or depolarized conditions in the presence or absence of 1 μ M AM251 ($n = 20$ fields from 4 independent experiments). All comparisons were significantly different ($p < 0.05$ with Bonferroni correction for multiple comparisons) except those indicated (N.S.). **G.** Summary of experiments measuring vGluT-1/FM1-43FX correspondence in neurons treated for 4 hr under baseline or depolarized conditions in the presence or absence of 200 nM DPCPX, 50 μ M SCH50911, 25 μ M LY341495, and 1 μ M AM251 (cocktail; $n = 20$ fields from 4 independent experiments). All comparisons were significantly different ($p < 0.05$ with Bonferroni correction for multiple comparisons) except those indicated (N.S.). **H.** Summary of experiments measuring EPSC amplitude in neurons treated 4 hr under baseline or depolarized conditions in the presence of either 1 μ M NBQX or 10 μ M NBQX to ensure kainate receptor block ($n = 14-16$ neurons). Neurons treated for 4 hr with 1 μ M NBQX were treated acutely (~ 1 min) with 10 μ M NBQX prior to recording as a control for any residual acute NBQX effects. All comparisons were significantly different ($p < 0.05$ with Bonferroni correction for multiple comparisons) except those indicated (N.S.).

To test G-protein-coupled CB1 cannabinoid receptor involvement, we incubated mass cultures in 1 μ M 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-1-piperidinyl-1H-pyrazole-3-carboxamide (AM251), a CB1 antagonist, during depolarizing or control challenge. To ensure that AM251 inhibited CB1 activation, we applied the CB1 agonist (R)-(+)-WIN55,212-2 (WIN; 200 nM) acutely to autaptic neurons. WIN acutely depressed PSCs by $34.6 \pm 8.6\%$ (Fig. 5E) while pre-incubation in AM251 prevented the WIN-induced depression ($101.1 \pm 2.5\%$ of baseline; $n = 8$ neurons per condition; Fig. 5E). AM251 incubation during the 4 hr depolarization challenge, however, altered neither the baseline percentage of active glutamatergic synapses nor depolarization-induced silencing (Fig. 5F). We conclude that CB1 activation does not participate in depolarization-induced presynaptic silencing.

It is plausible that multiple GPCRs are simultaneously activated by strong depolarization. If this occurs, then each receptor might contribute individually to a small, statistically insignificant effect on the percentage of active presynaptic terminals. Simultaneous activation, however, may synergistically upregulate the activity of downstream signaling cascades. To test this hypothesis, we incubated mass cultures for 4 hr in a cocktail of GPCR antagonists (200 nM DPCPX, 50 μ M SCH50911, 25 μ M LY341495, and 1 μ M AM251) to simultaneously block the activation of A1 adenosine receptors, GABA_B receptors, mGluRs, and CB1 receptors. This antagonist cocktail did not alter the percentage of active glutamatergic presynaptic terminals at baseline (Fig. 5G), indicating that transmitter tone over 4 hr was not sufficient to induce additional muting. Additionally, this cocktail of GPCR antagonists did not prevent silencing in response to depolarization (Fig. 5G). Interestingly, we failed to observe the trend-level

increase in the percentage of active synapses produced in depolarized neurons exposed to DPCPX (Fig. 4D). This suggests that these four receptor classes, although linked to inhibitory G-proteins, are not responsible for the induction of depolarization-induced presynaptic silencing.

Although 1 μ M NBQX was present during all depolarization challenges to prevent ionotropic glutamate receptor activation, kainate receptors may not be blocked at this concentration (Bureau et al., 1999; Crowder et al., 2002). Kainate receptors can induce metabotropic effects dependent on G-proteins in addition to their ionotropic effects (Cunha et al., 1999; Lerma, 2003; Rodriguez-Moreno and Sihra, 2007). To ensure that kainate receptors and their downstream metabotropic signaling cascades are not activated during 4 hr depolarization, we co-incubated with 10 μ M NBQX, which should block both kainate receptors and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Lauri et al., 2001; Delaney and Jahr, 2002). After a 4 hr depolarizing or control challenge, we recorded EPSC amplitudes in autaptic neurons. Control cultures were treated acutely for 1 min with 10 μ M NBQX just before recording to account for any residual effects of the high NBQX concentration on AMPA receptors. The higher concentration of NBQX did not prevent depolarization-induced silencing and did not alter the EPSC amplitude compared with control neurons (Fig. 5H). This suggests that metabotropic effects downstream of kainate receptor activation are unimportant for depolarization-induced silencing.

A1 receptor activation and GABA_B receptor activation induce presynaptic silencing:

It is intriguing that A1 receptor, GABA_B receptor, mGluR, CB1, and kainate

receptor activation are not necessary for induction of presynaptic silencing despite our evidence that depolarization-induced silencing depends on pertussis toxin-sensitive G-proteins. These results suggest that inhibitory G-proteins are activated during strong depolarization but not by activation of these particular receptors. To further test our hypothesis that a G-protein-dependent mechanism, regardless of the source of activation, controls adaptive presynaptic silencing, we tested whether direct activation of several GPCR classes induces silencing.

We first determined whether increases in exogenous extracellular adenosine concentration induce presynaptic silencing. We incubated cultures in the presence of 100 μ M adenosine for 4 hr. A high concentration was chosen to counteract any degradation that may occur from the natural activity of extracellular enzymes or uptake through transporters (Dunwiddie and Masino, 2001). We used FM1-43FX loading of presynaptic terminals followed by vGluT-1 immunostaining to determine the percentage of active glutamatergic terminals. Incubation for 4 hr with 100 μ M adenosine decreased the percentage of active synapses (Fig. 6A, B). To determine if this effect of adenosine resulted from activation of the A1 receptor, we co-incubated with DPCPX (200 nM). DPCPX incubation alone did not alter the percentage of active glutamatergic synapses, but it prevented the adenosine-induced silencing (Fig. 6A, B). This result also serves as an effective positive control for experiments testing DPCPX effects on depolarization-induced muting (Fig. 4). Note that FM1-43FX uptake should be immune to the well-known transient, acute effects of adenosine agonists on p_r since agonists and antagonists were washed out of the cultures before the FM1-43FX challenge (Wu and Saggau, 1994; Brown and Sihra, 2008). Also, FM1-43FX loading was conducted during 2 min of a

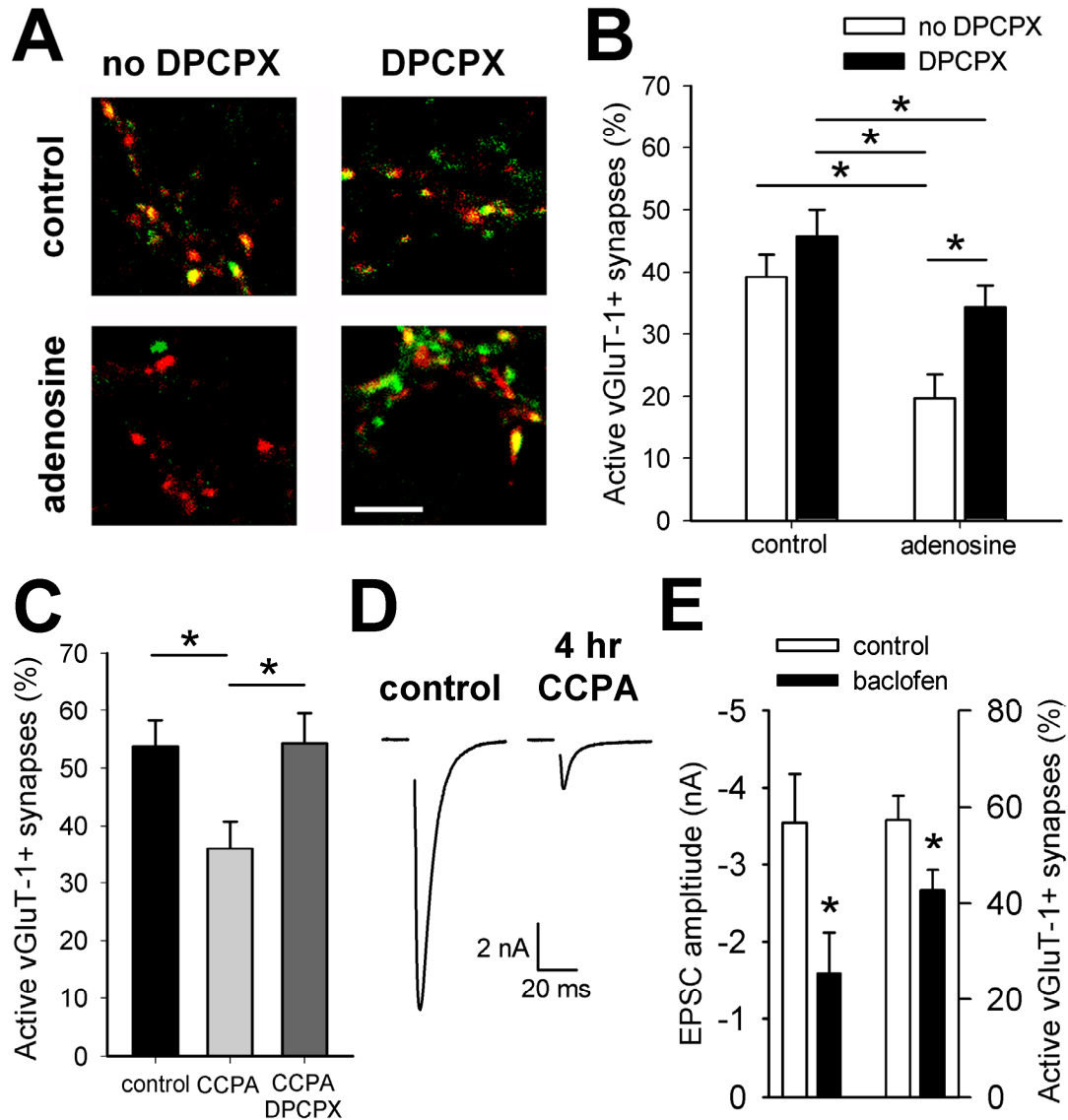


Figure 6. A1 adenosine receptor activation and GABA_B receptor activation induce presynaptic silencing. **A.** Example images of vGluT-1 (red) and FM1-43FX (green) correspondence in neurons treated 4 hr with control or 100 μ M adenosine in the presence or absence of 200 nM DPCPX. Scale bar denotes 5 μ M. **B.** Summary of conditions represented in A ($n = 40$ fields from 8 independent experiments). $*p < 0.05$ with Bonferroni correction for multiple comparisons. **C.** Summary of experiments measuring vGluT-1/FM1-43FX correspondence in neurons treated 4 hr with control medium, with 10 nM CCPA, or with 10 nM CCPA co-applied with 200 nM DPCPX ($n = 35$ fields from 7 independent experiments). $*p < 0.05$ with Bonferroni correction for multiple comparisons. **D.** Example action potential-evoked EPSCs recorded from autaptic neurons after acute (< 1 min; control) or 4 hr treatment with 10 nM CCPA. **E.** Summary of EPSC amplitudes from autaptic neurons treated acutely (< 1 min; control) or for 4 hr with 50 μ M baclofen ($n = 9-10$ neurons) and FM1-43FX/vGluT-1 correspondence after 4 hr control or 50 μ M baclofen treatment ($n = 30$ fields from 6 independent experiments). $*p < 0.05$.

strong depolarization designed to empty the entire recycling pool of vesicles regardless of p_r . Thus, these results suggest that extracellular adenosine induces presynaptic silencing through an A1 receptor-dependent mechanism.

Results with adenosine do not exclude a contribution from A2 or A3 receptors, although this contribution is presumably minimal because the A1-specific antagonist DPCPX blocked the silencing. To confirm that A1 receptor activation alone is sufficient for presynaptic silencing induction, we incubated mass cultures in the presence of the highly selective A1 receptor agonist CCPA. CCPA (10 nM for 4 hr) decreased the percentage of active glutamatergic terminals, and the CCPA effect was blocked by co-incubation with 200 nM DPCPX (Fig. 6C). We also confirmed that CCPA persistently depressed presynaptic action potential-driven vesicle release by measuring autaptic EPSC amplitudes after 4 hr of CCPA incubation. Electrophysiological recordings were performed in extracellular recording saline in the absence of CCPA, so acute effects are unlikely. However, to ensure that EPSC depression was not caused by residual acute effects of CCPA, a high-affinity ligand which might have failed to dissociate from A1 receptors during the media exchange (Lohse et al., 1988), we used neurons from sibling cultures acutely exposed to 10 nM CCPA (< 1 min) as our control. EPSC amplitudes were significantly decreased in neurons recorded after 4 hr treatment with CCPA compared to those treated acutely with CCPA (control: -8.92 ± 1.19 nA; 4 hr CCPA: -2.59 ± 1.10 nA; $n = 10$; $p = 0.001$; Fig. 6D). Additionally, we measured paired-pulse depression in order to assess if lingering CCPA altered vesicle p_r . An increased paired-pulse ratio (e.g. a decreased paired-pulse depression) is typically interpreted to reflect decreased p_r , which could be caused by presynaptic GPCR activation (Mennerick and

Zorumski, 1995; Dobrunz and Stevens, 1997; Zucker and Regehr, 2002; Brown and Sihra, 2008). The paired-pulse depression in neurons treated acutely with CCPA ($23.4 \pm 5.5\%$; $n = 10$) was not significantly different from the depression in neurons treated for 4 hr ($25.8 \pm 7.0\%$; $n = 9$; $p = 0.79$; supplemental Fig. 1C). Together, these results suggest a novel presynaptic muting by prolonged selective activation of A1 adenosine receptors. Presynaptic silencing outlives agonist exposure and does not result from altered p_r .

We next incubated neurons in 50 μM baclofen, the GABA_B receptor agonist, for 4 hr before evaluation in baclofen-free solutions. Baclofen, in the absence of any depolarizing stimulus, persistently depressed autaptic EPSC amplitudes and reduced the percentage of active glutamatergic presynaptic terminals (Fig. 6E). Baclofen did not, however, have a persisting effect on the paired-pulse depression (control: $20.8 \pm 7.3\%$; baclofen: $1.1 \pm 7.7\%$; $n = 9$; $p = 0.08$; supplemental Fig. 1D). Additionally, we have confirmed that the effects of CCPA and baclofen on FM1-43FX/vGluT-1 correspondence do not result from transient actions of these agonists. We co-applied CCPA and baclofen for 4 hr and allowed 10 min of recovery in untreated media before FM1-43FX loading. Neurons treated with CCPA and baclofen maintained a decreased percentage of active glutamatergic presynaptic terminals after this period of recovery (control + recovery: $79.0 \pm 4.3\%$; CCPA/baclofen + recovery: $51.5 \pm 4.5\%$; $n = 20$ fields from 4 coverslips; $p = 0.00009$). In contrast, saturating concentrations of neither ACPD nor another mGluR agonist L-AP4 induced silencing (supplemental Fig. 3A, B). Similarly, CB1 agonist WIN did not induce silencing at a saturating concentration (supplemental Fig. 3C), and kainate receptor agonist kainic acid did not produce silencing at concentrations of 1 μM or 10 μM (supplemental Fig. 3D, E). These results suggest that GPCR-induced silencing

is induced by only a select group of receptors. The lack of silencing induced by mGluR and CB1 activation may be caused, for example, by low receptor expression levels in presynaptic terminals of cultured hippocampal neurons (as suggested by the weak acute effects of near-saturating agonist concentrations in Figure 5C, E) or by divergent downstream signaling cascades.

GPCR-induced silencing is proteasome-dependent:

Inhibitory G-protein activation links depolarization-induced silencing and GPCR agonist-induced silencing, but it is unclear whether GPCR-dependent presynaptic silencing invokes similar downstream signaling cascades as depolarization-induced presynaptic silencing. For instance, maximum GPCR agonist-induced silencing tends to be weaker than depolarization-induced silencing (see Fig. 6C, E), which could hint at different underlying mechanisms. It was recently shown that depolarization-induced muting is ultimately dependent on activity of the ubiquitin proteasome system (UPS). Depolarization-induced presynaptic silencing is prevented by co-incubation with the proteasome inhibitor MG-132 (Jiang et al., 2010). To test whether GPCR agonist-induced silencing also recruits the UPS, we blocked proteasome activity by 30 min pre-incubation with MG-132 alone before co-applying MG-132 with GPCR agonists for 4 hr. To maximize the inhibitory G-protein signal, we co-applied 10 nM CCPA and 50 μ M baclofen to simultaneously activate both A1 receptors and GABA_B receptors. This treatment did not result in more silencing than either agonist alone (compare Fig. 6C, D, E with Fig. 7) as expected from convergent downstream signaling in the two receptor systems (Brown and Sihra, 2008). MG-132 and CCPA/baclofen treatment did not alter

paired-pulse depression in autaptic neurons (control: $21.1 \pm 4.0\%$; MG-132: $24.3 \pm 5.7\%$; CCPA/baclofen: $22.3 \pm 5.8\%$; CCPA/baclofen + MG-132: $24.7 \pm 7.9\%$; $n = 14-15$ neurons per condition; $p > 0.05$; also see supplemental Fig. 1E). To confirm that a p_r change should have been detected by paired-pulse measurements with our protocols and sample sizes, we tested the effect of acute $10 \mu\text{M}$ baclofen application on EPSC paired-pulse depression. As expected, paired-pulse modulation was significantly altered by acute baclofen ($3.7 \pm 5.2\%$ depression in saline control; $32.3 \pm 15.6\%$ facilitation in baclofen; $n = 12$; $p = 0.04$; supplemental Fig. 1F). MG-132 alone did not alter the baseline EPSC amplitude, but it prevented silencing normally induced by GPCR agonist application (Fig. 7A, B). This was confirmed with FM1-43FX labeling of presynaptic terminals. The percentage of active glutamatergic presynaptic terminals was not altered by MG-132 alone, but co-incubation with GPCR agonists prevented presynaptic silencing (Fig. 7C, D). These results suggest that GPCR agonist-induced silencing also recruits a proteasome-dependent mechanism, supporting our hypothesis that persistent GPCR-dependent depression utilizes similar signaling cascades as depolarization-induced silencing. This also supports our model that depolarization-induced silencing occurs through a calcium-independent inhibitory G-protein-dependent mechanism that ultimately depends on activation of the UPS.

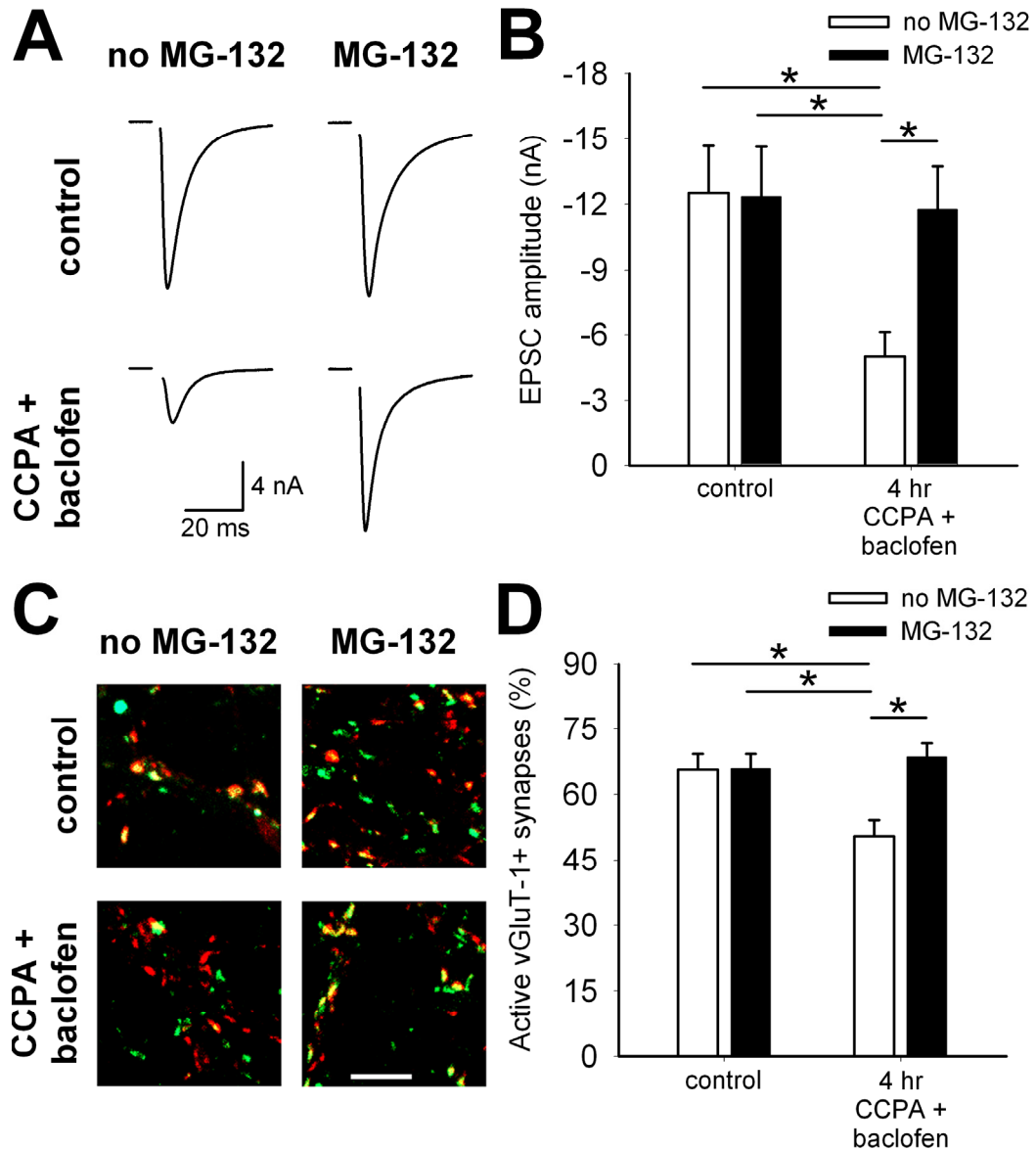


Figure 7. GPCR-dependent silencing requires proteasome activity. **A.** Example action potential-evoked EPSCs from autaptic neurons treated acutely (< 1 min; control) or for 4 hr with 10 nM CCPA and 50 μ M baclofen with or without 3 μ M MG-132. MG-132 was added 30 min prior to the start of (and remained during) the 4 hr treatment. **B.** Summary of EPSC amplitudes from autaptic neurons treated as in A. $*p < 0.05$ with Bonferroni correction for multiple comparisons ($n = 15$ neurons). **C.** Example images of vGluT-1 (red) and FM1-43FX (green) correspondence in neurons treated 4 hr with or without 10 nM CCPA and 50 μ M baclofen in the presence or absence of 3 μ M MG-132. MG-132 was added 30 min prior to the start of the 4 hr treatment. Scale bar denotes 5 μ m. **D.** Summary of experiments measuring vGluT-1/FM1-43FX correspondence in neurons treated as in C. $*p < 0.05$ with Bonferroni correction for multiple comparisons ($n = 35$ fields from 7 independent experiments).

Discussion

Adaptive modulation at synapses has been studied extensively, but induction mechanisms remain unclear (see Pozo and Goda, 2010). We tested whether calcium and G-protein signaling, two potential upstream regulators of cAMP, are necessary for presynaptic muting. Surprisingly, depolarization-induced presynaptic silencing is not dependent on calcium rises from extracellular or intracellular sources. Depolarization-induced muting is pertussis toxin-sensitive, implicating $G_{i/o}$ signaling, and prolonged, direct activation of two classes of $G_{i/o}$ -linked GPCRs induces silencing, suggesting novel presynaptic $G_{i/o}$ -induced modulation. Blocking five receptor classes failed to prevent depolarization-induced silencing, suggesting that G-protein signaling is activated by a pathway that remains untested. Furthermore, GPCR-dependent silencing is proteasome-dependent, as previously documented for depolarization-induced silencing. Our results support the model that prolonged depolarization upregulates inhibitory G-protein signaling, possibly independent of receptor activation, to ultimately degrade vital regulators of neurotransmission.

Calcium and persistent forms of synaptic plasticity:

Calcium from neither the extracellular space (Fig.1) nor intracellular stores (Fig. 2) is necessary for adaptive presynaptic silencing. This result is surprising because multiple persistent forms of hippocampal synaptic plasticity depend on calcium (Dunwiddie et al., 1978; Wigstrom et al., 1979; Lynch et al., 1983; Williams and Johnston, 1989; Wickens and Abraham, 1991; Mulkey and Malenka, 1992; Xie et al., 1992; Tzounopoulos et al., 1998; Patenaude et al., 2003; Kellogg et al., 2009). Other

adaptive forms of plasticity, including synaptic scaling and plasticity of intrinsic excitability, require changes in intracellular calcium (Thiagarajan et al., 2002; Cudmore and Turrigiano, 2004; Frank et al., 2006; Ibata et al., 2008; Wu et al., 2008). Although it is possible that depolarization-induced presynaptic silencing requires postsynaptic depolarization, our BAPTA-AM experiments exclude an important induction role for any source of calcium, including presynaptic, postsynaptic, and glial sources. These results are consistent with our previous finding that calcium-sensitive adenylyl cyclase isoforms play no role in silencing induction (Moulder et al., 2008).

Depolarization-induced muting is among a few examples of calcium-independent plasticity. These include short-term, frequency-dependent synaptic depression (Betz, 1970; Stevens and Tsujimoto, 1995; Garcia-Perez et al., 2008), transient modulation by non-traditional messengers like nitric oxide and steroid hormones (Meffert et al., 1996; Zadran et al., 2009), and some forms of hippocampal mGluR-dependent long-term depression (Fitzjohn et al., 2001; Ireland and Abraham, 2009). Because none of these mechanisms is implicated in presynaptic muting, the silencing described herein appears to be unique.

Presynaptic inhibition by G-protein activation:

Acute activation of pertussis toxin-sensitive presynaptic and postsynaptic inhibitory G-proteins induces well-known, rapidly reversible effects on calcium currents and inwardly rectifying potassium channels (Brown and Sihra, 2008). Although acute presynaptic depression elicited by $G_{i/o}$ stimulation persists for at least 4 hr in the continued presence of agonist (Wetherington and Lambert, 2002a, b), several major

features distinguish the depolarization-induced and GPCR-induced presynaptic silencing observed here from this classical presynaptic GPCR-induced depression.

First, acute $G_{i/o}$ activation causes synaptic depression with rapid onset and rapid offset upon agonist withdrawal (Brown and Sihra, 2008). Presynaptic muting, in contrast, requires prolonged stimulation, and recovery requires hours (Moulder et al., 2004). GPCR agonist-induced silencing failed to recover within 10 min in imaging experiments and persisted for at least an hour after agonist removal in our electrophysiology experiments (Fig. 6, 7). Furthermore, only 2 of 5 receptor classes tested induced presynaptic silencing. It is possible that persistent silencing requires stronger $G_{i/o}$ stimulation than acute depression, since CB1 and mGluR stimulation yielded weak acute depression and no detectable silencing (Fig. 5C, E and supplemental Fig. 3).

Second, acute depression depends on $G_{\beta\gamma}$ -dependent presynaptic inhibition of calcium channels to reduce vesicle p_r (Brown and Sihra, 2008). Decreases in p_r are reflected as increased paired-pulse ratios (Dobrunz and Stevens, 1997; Zucker and Regehr, 2002). Depolarization-induced and GPCR-induced muting did not alter paired-pulse ratios (supplemental Fig. 1) and may involve degradation of important presynaptic proteins through the UPS (Jiang et al., 2010). This is an unusual profile for presynaptic change and might explain why GPCR-induced silencing has been overlooked. Thus, persistent GPCR-dependent presynaptic silencing invokes different mechanisms than acute GPCR-dependent synaptic depression.

Other forms of slowly-induced synaptic depression dependent on GPCRs have been described in the hippocampus. For example, GPCR activation is necessary for

certain forms of long-term depression or depotentiation of long-term potentiation (Oliet et al., 1997; Palmer et al., 1997; Chevaleyre and Castillo, 2004; Izumi and Zorumski, 2008; Kellogg et al., 2009). Although a presynaptic change has been implicated in some of these examples, presynaptic muting has not been implicated because these are calcium-dependent and/or require activation of receptors that we have shown are unnecessary for depolarization-induced presynaptic silencing.

The finding that prolonged A1 and GABA_B receptor activation induces silencing is reminiscent of evidence for tonic GPCR activation *in situ*. A1 receptors and GABA_B receptors are constitutively active at low levels under physiological conditions (Canning and Leung, 2000; Thummler and Dunwiddie, 2000; Jensen et al., 2003; Kukley et al., 2005; but see Ariwodola and Weiner, 2004). Thus, GPCR-induced silencing may contribute to basally silent terminals *in situ*. Evidence for basal GPCR contributions to presynaptically mute synapses in our cultures was limited (Fig. 5B), possibly because of much larger dilution of transmitters than occurs *in situ*. Because evoked accumulation of adenosine and GABA likely achieves higher local concentrations over more prolonged periods in intact tissue (Mitchell et al., 1993; Masino and Dunwiddie, 1999; de Groote and Linthorst, 2007; Ghijssen et al., 2007), GPCR-induced silencing could play a dynamic role in sculpting the pattern of silent and active terminals.

Relationship between G-proteins and proteasome activity:

Multiple downstream signaling cascades are activated by GPCRs. Our data implicate that, ultimately, GPCR agonist-induced presynaptic silencing is dependent upon activity of the UPS since the proteasome inhibitor MG-132 blocked silencing

induction (Fig. 7). One potential model is that inhibition of adenylyl cyclase by G-proteins decreases cAMP accumulation and associated protein phosphorylation by protein kinase A. We speculate that phosphorylation may protect key presynaptic proteins from degradation by the UPS. This view is consistent with evidence that downregulation of cAMP signaling leads to silencing (Moulder et al., 2008) and that depolarization-induced silencing is proteasome-dependent (Jiang et al., 2010). In addition Munc13-1 and Rim1 α , presynaptic proteins essential for vesicle priming (Augustin et al., 1999; Betz et al., 2001; Koushika et al., 2001), are selectively degraded relative to other presynaptic proteins after stimuli that induce presynaptic silencing (Jiang et al., 2010). This mirrors work in *Drosophila* showing that the Munc13 ortholog Dunc13 is regulated in a G-protein-dependent, cAMP-dependent, and proteasome-dependent manner (Aravamudan and Broadie, 2003) as well as work at the calyx of Held showing that GABA_B receptor activation inhibits vesicle recruitment via reduced cAMP levels (Sakaba and Neher, 2003). Since Rim1 α is a protein kinase A and UPS substrate (Lonart et al., 2003; Yao et al., 2007), phosphorylation-induced Rim1 α stability could link increased cAMP levels to preserved presynaptic function. This speculative scenario does not preclude the possibility that induction stimuli modulate proteasome activity directly in a cAMP-independent manner (Jiang et al., 2010). Future work may clarify whether cAMP-independent G-protein signaling upregulates proteasome activity or whether depolarization increases proteasome activity by a pathway divorced from G-protein signaling.

G-protein signaling in depolarization-induced silencing:

Although depolarization-induced presynaptic silencing requires activation of inhibitory G-proteins, how G-proteins are activated during prolonged depolarization remains unclear. We excluded four primary candidate GPCRs as well as metabotropic actions of kainate receptors (Fig. 4, 5). Depolarization may cause release of a ligand for $G_{i/o}$ -linked GPCRs that we did not test. For example, neuromodulatory GPCRs like dopamine and serotonin receptors are possible candidates (Catapano and Manji, 2007), but monoaminergic cells are absent from our cultures, making ligand presence extremely unlikely. Furthermore the calcium independence of presynaptic muting excludes an important role of synaptic release of these potential mediators. Clearly, if a ligand is involved in induction of depolarization-induced muting, it is released through a non-classical mechanism. Alternatively, cell autonomous mechanisms such as voltage-sensitive proteins, including inhibitory GPCRs activated directly by voltage, could mediate silencing induction (Reddy et al., 1995; Ben-Chaim et al., 2006; Parnas and Parnas, 2007; Okamura et al., 2009). One intriguing possibility is that of receptor-independent activation of G-proteins, for instance through recently-described activators of G-protein signaling (AGS; Blumer et al., 2007). AGS proteins interact with G-protein subunits to initiate downstream signaling in the absence of receptor activation. Some AGS proteins are expressed in hippocampal neurons, but it is unclear how they are activated (Fang et al., 2000; Blumer et al., 2002; Takahashi et al., 2003).

Although we have learned much about the induction of presynaptic silencing in excitatory neurons (Tong et al., 1996; Ma et al., 1999; Moulder et al., 2004; Voronin and Cherubini, 2004; Atasoy and Kavalali, 2006; Moulder et al., 2006; Yao et al., 2006; Moulder et al., 2008; Jiang et al., 2010), many segments of the signaling cascade have

remained obscure. We show here that calcium-independent, G-protein-dependent, and proteasome-dependent mechanisms induce presynaptic muting, suggesting a unique form of synaptic plasticity. Because adaptive synaptic plasticity may counteract potentially damaging changes in neuronal activity (Turrigiano and Nelson, 2004), further clarification of silencing induction pathways may lead to new therapeutic strategies.

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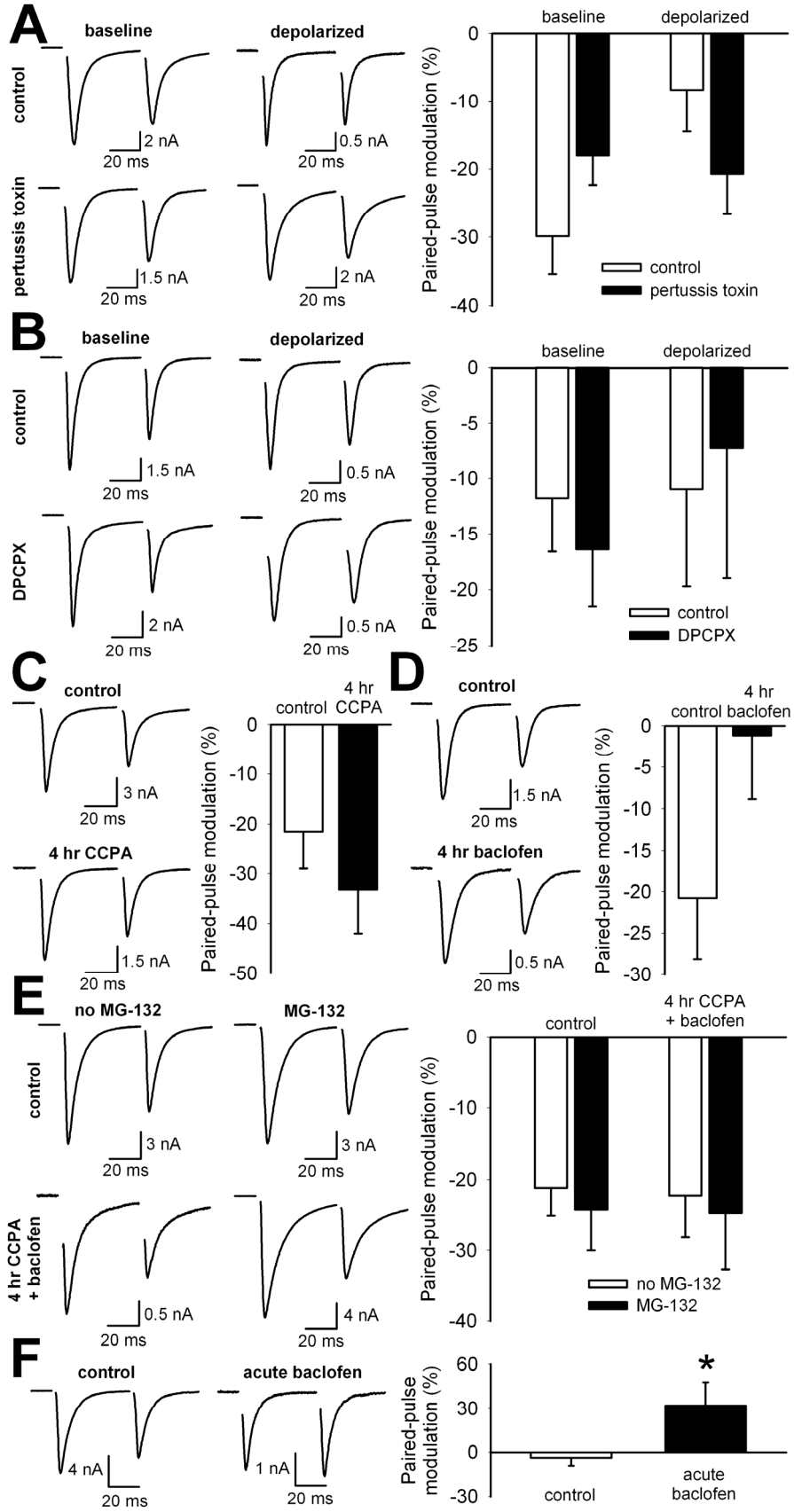
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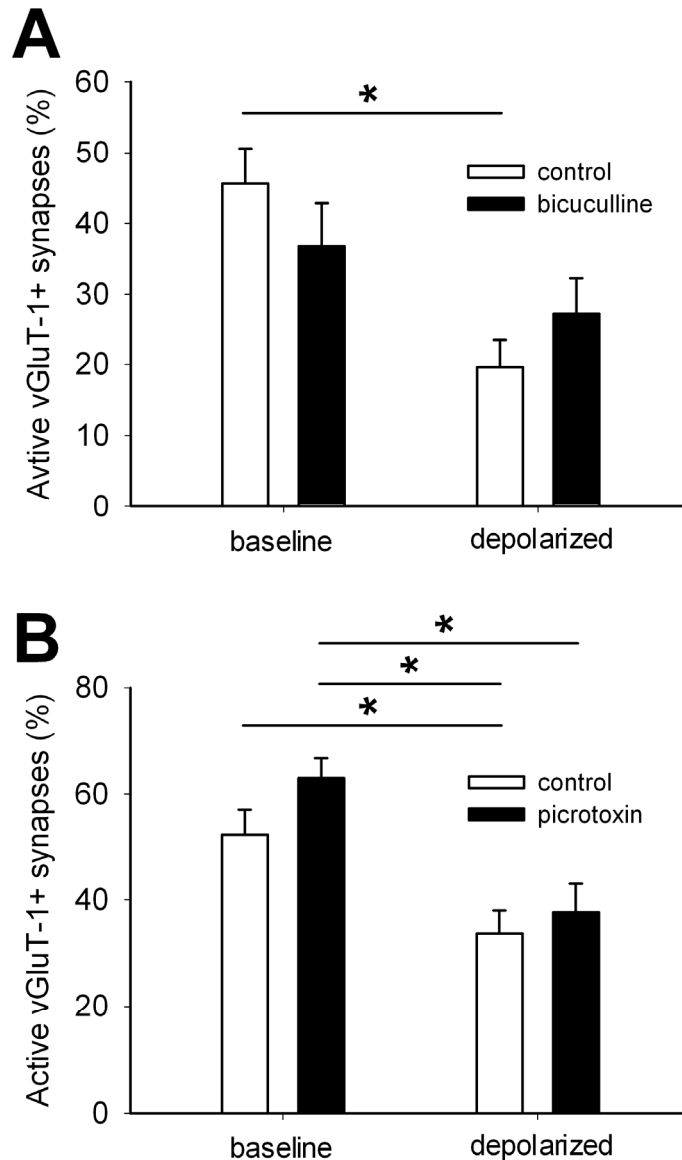
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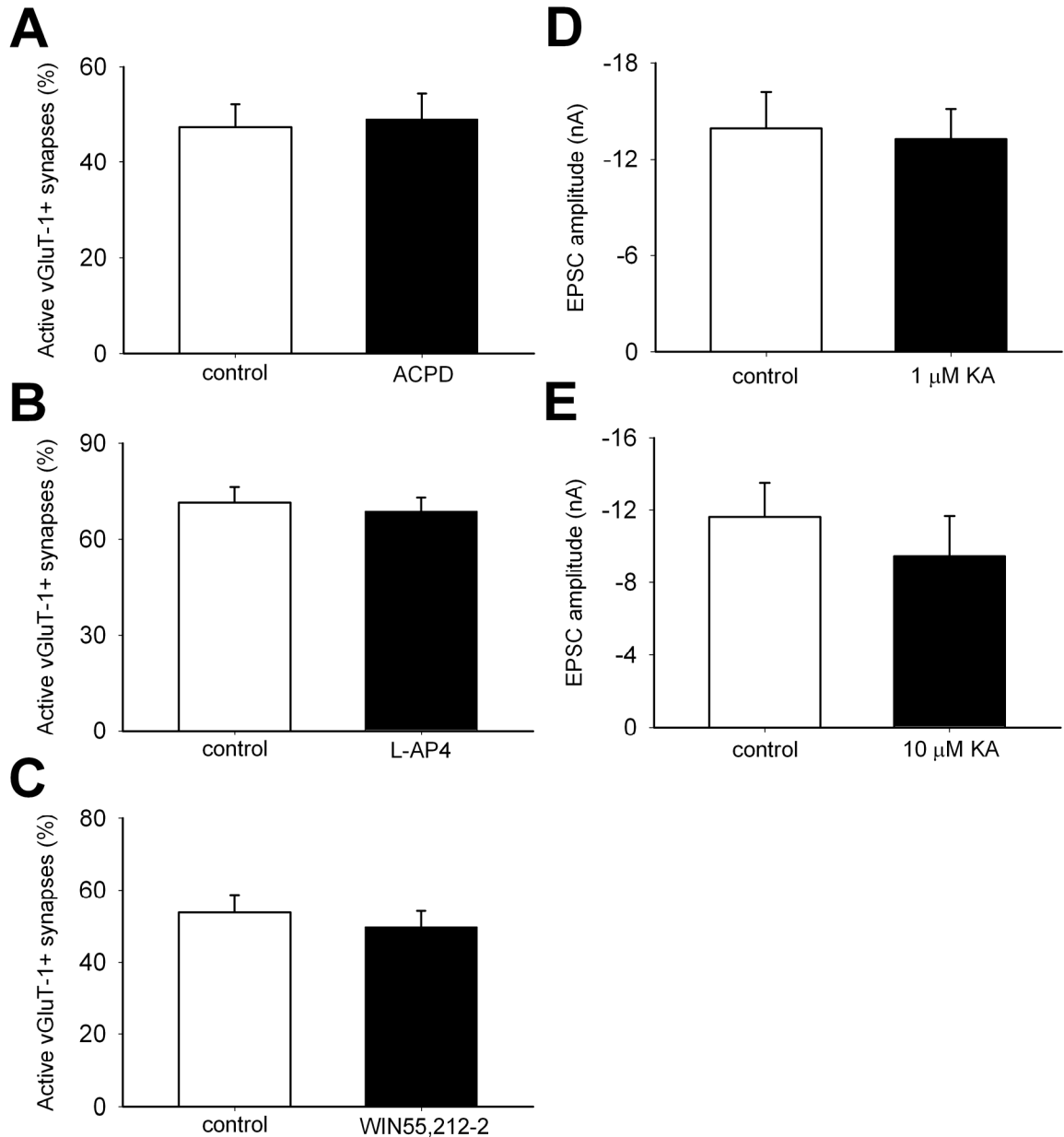
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Supplemental Figure 1. Acute, but not prolonged, treatments alter paired-pulse modulation in excitatory autaptic neurons. **A.** Left panel: example action-potential evoked EPSCs with 50 ms interstimulus interval from autaptic neurons treated 24 hr with or without 500 ng/ml pertussis toxin before 4 hr co-application with 30 mM NaCl (baseline) or 30 mM KCl (depolarized). Right panel: summary of percentage change in EPSC amplitude during 50 ms paired-pulse stimulus in neurons treated as described in the left panel. $p = 0.09-1.00$ with Bonferroni correction for multiple comparisons ($n = 13-14$). **B.** Left panel: example action-potential evoked EPSCs with 50 ms interstimulus interval from autaptic neurons treated 4 hr with 30 mM NaCl (baseline) or 30 mM KCl (depolarized) in the presence or absence of 200 nM DPCPX. Right panel: summary of percentage change in EPSC amplitude during 50 ms paired-pulse stimulus in neurons treated as described in the left panel. $p = 0.59-1.00$ with Bonferroni correction for multiple comparisons ($n = 9$). **C.** Left panel: example action-potential evoked EPSCs with 50 ms interstimulus interval from autaptic neurons treated either acutely (< 1 min; control) or for 4 hr with 10 nM CCPA. Right panel: summary of percentage change in EPSC amplitude during 50 ms paired-pulse stimulus in neurons treated as described in the left panel. $p = 0.79$ ($n = 9-10$). **D.** Left panel: example action-potential evoked EPSCs with 50 ms interstimulus interval from autaptic neurons treated either acutely (< 1 min; control) or for 4 hr with 50 μ M baclofen. Right panel: summary of percentage change in EPSC amplitude during 50 ms paired-pulse stimulus in neurons treated as described in the left panel. $p = 0.08$ ($n = 9$). **E.** Left panel: example action-potential evoked EPSCs with 50 ms interstimulus interval from autaptic neurons treated acutely (< 1 min; control) or for 4 hr with 10 nM CCPA and 50 μ M baclofen with or without 3 μ M MG-132. MG-132 was added 30 min prior to the start of (and remained during) the 4 hr treatment. Right panel: summary of percentage change in EPSC amplitude during 50 ms paired-pulse stimulus in neurons treated as described in the left panel. $p = 0.66-0.97$ without correction for multiple comparisons ($n = 14-15$). **F.** Left panel: example action-potential evoked EPSCs with 50 ms interstimulus interval from autaptic neurons treated acutely with locally perfused saline control or 10 μ M baclofen. Right panel: summary of percentage change in EPSC amplitude during 50 ms paired-pulse stimulus in neurons treated as described in the left panel. $*p = 0.04$ ($n = 12$).



Supplemental Figure 2. Blocking GABA_A receptors does not prevent depolarization-induced silencing. **A.** Summary of experiments measuring vGluT-1/FM1-43FX correspondence in neurons treated 4 hr with 30 mM NaCl (baseline) or 30 mM KCl (depolarized) in the presence or absence of 50 μ M bicuculline, a GABA_A receptor antagonist. $*p < 0.05$ with Bonferroni correction for multiple comparisons ($n = 25$ fields from 5 independent experiments). **B.** Summary of experiments measuring vGluT-1/FM1-43FX correspondence in neurons treated 4 hr with 30 mM NaCl (baseline) or 30 mM KCl (depolarized) in the presence or absence of 100 μ M picrotoxin, another GABA_A receptor antagonist. $*p < 0.05$ with Bonferroni correction for multiple comparisons ($n = 30$ fields from 6 independent experiments).



Supplemental Figure 3. mGluR, CB1, and kainate receptor agonists do not induce silencing. **A.** Summary of experiments measuring vGluT-1/FM1-43FX correspondence in neurons treated 4 hr with or without 200 μ M ACPD (mGluR agonist; $n = 30$ fields from 6 coverslips; $p = 0.81$). **B.** Summary of experiments measuring vGluT-1/FM1-43FX correspondence in neurons treated 4 hr with or without 10 μ M L-AP4 (mGluR agonist; $n = 15$ fields from 3 coverslips; $p = 0.69$). **C.** Summary of experiments measuring vGluT-1/FM1-43FX correspondence in neurons treated 4 hr with or without 1 μ M WIN55,212-2 (CB1 agonist; $n = 40$ fields from 8 coverslips; $p = 0.54$). **D.** Summary of experiments measuring EPSC amplitude in neurons treated 4 hr with or without 1 μ M kainic acid (KA; kainate receptor agonist; $n = 12$ neurons; $p = 0.82$). **E.** Summary of experiments measuring EPSC amplitude in neurons treated 4 hr with or without 10 μ M kainic acid ($n = 11$ neurons; $p = 0.47$).

Chapter 4

Astrocyte-derived thrombospondins mediate the development of hippocampal presynaptic plasticity

This chapter contains a manuscript submitted for publication:

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Author contributions for the citation above:

D.C.C. designed and performed experiments, analyzed data, and wrote the paper. S.M. designed experiments, performed experiments for Figure 3, analyzed data, and wrote the paper. X.J. performed microscopy experiments and analyzed data. A.T. analyzed data.

Abstract

Astrocytes contribute to many neuronal functions, including synaptogenesis, but their role in the development of synaptic plasticity remains unclear. Presynaptic muting of hippocampal glutamatergic terminals defends against excitotoxicity and has been demonstrated at diverse synaptic terminals. Here we studied the role of astrocytes in the development of presynaptic muting at glutamatergic hippocampal synapses. We found that astrocytes were critical for the development of depolarization-dependent and $G_{i/o}$ -dependent presynaptic muting. The ability of cAMP analogues to modulate presynaptic function was also impaired by astrocyte deficiency. Although astrocyte deprivation resulted in postsynaptic glutamate receptor deficits, this effect appeared independent of astrocytes' role in presynaptic muting. Muting was restored with chronic but not acute treatment with astrocyte-conditioned medium, indicating that a soluble factor is permissive for muting. Astrocyte-derived thrombospondins (TSPs) are likely responsible because TSP1 mimicked the effect of conditioned medium, and gabapentin, a high-affinity antagonist of TSP binding to the $\alpha 2\delta$ -1 calcium channel subunit, mimicked astrocyte deprivation. We found evidence that PKA activity is abnormal in astrocyte-deprived neurons but restored by TSP1, so PKA dysfunction may provide a mechanism by which muting is disrupted during astrocyte deficiency. In summary our results suggest an important role for astrocyte-derived TSPs, acting through $\alpha 2\delta$ -1, in maturation of a potentially important form of presynaptic plasticity.

Introduction

Correct development and plasticity of glutamate synapses is critical to brain function. For example, imbalances between excitatory glutamate and inhibitory GABA actions are postulated to underlie brain disturbances as diverse as schizophrenia (Lewis and Moghaddam, 2006), developmental disability (Wetmore and Garner, 2010), and epilepsy (McCormick and Contreras, 2001). Diverse cues regulate proper synaptic development. Neuron-to-neuron signaling is clearly important for synaptogenesis (Craig et al., 2006), but astrocytic factors are also important (Eroglu and Barres, 2010; Pfrieger, 2010). Still unclear is the breadth of astrocyte involvement in synapse development and the signaling systems affected. Astrocytic cues can affect postsynaptic function, presynaptic function, synaptic plasticity, or combinations of these elements.

Our group and others have studied a form of adaptive presynaptic plasticity termed muting whereby the number of terminals releasing transmitter is reduced. Mute presynaptic terminals have been studied in a variety of preparations (Tong et al., 1996; Bolshakov et al., 1997; Kannenberg et al., 1999; Ma et al., 1999; Kim et al., 2003; Losonczy et al., 2004; Moulder et al., 2004; Cousin and Evans, 2011; Crawford and Mennerick, 2012). Reversible presynaptic muting of hippocampal glutamatergic synapses occurs in cultured autaptic neurons, conventional mass cultures, and acute hippocampal slices (Moulder et al., 2004; Crawford et al., 2011). Muting is induced by electrical stimulation, depolarization challenges, prolonged $G_{i/o}$ G-protein activation, and hypoxic environmental exposures (Moulder et al., 2004; Moulder et al., 2006; Moulder et al., 2008; Crawford et al., 2011; Hogins et al., 2011; Crawford and Mennerick, 2012). Presynaptic muting may play a role in defending against excitotoxic glutamate release

(Hogins et al., 2011), but it is also engaged by physiological changes in activity (Moulder et al., 2006). Despite the wide variety of preparations and conditions under which muting occurs (Crawford and Mennerick, 2012), factors controlling development of muting have not been explored.

Muting occurs in single-neuron networks in culture, but because these neurons contact astrocytes it is unclear whether muting is cell autonomous or whether astrocyte signals modulate or induce muting. Here we explored muting mechanisms in a reductionist environment where astrocyte numbers could be reduced. We found that global, permissive astrocytic signaling rather than local, instructive astrocytic signaling is important for the development of muting. Astrocyte deprivation prevented muting in response to both depolarization and G-protein-coupled receptor stimulation. Chronic, but not acute, treatment with astrocyte-conditioned medium rescued muting.

Thrombospondin (TSP), an astrocyte-derived glycoprotein previously implicated in glutamate synapse development (Asch et al., 1986; Christopherson et al., 2005; Eroglu et al., 2009; Xu et al., 2010), also rescued muting. Gabapentin, an antagonist of the TSP receptor $\alpha 2\delta$ -1 (Gee et al., 1996; Eroglu et al., 2009), mimicked the effects of astrocyte deprivation by preventing muting competence. Gabapentin additionally prevented adaptive network plasticity. Although the downstream pathways initiated by TSP binding to $\alpha 2\delta$ -1 remain unclear, astrocyte deprivation led to altered PKA activity, as suggested by abnormal responses to cAMP analogues and over-phosphorylation of PKA substrates. TSP treatment corrected these abnormalities. Our results suggest that astrocyte-derived TSPs are important developmental modulators of presynaptic plasticity.

Materials and Methods

Primary Hippocampal Cultures:

All experiments were performed in accordance with National Institutes of Health guidelines and were approved by the Washington University Animal Studies Committee. For microcultures, culture dishes (35 mm) were first covered with a thin layer of 0.15% agarose and then stamped with 150-200 μm diameter microdots of 0.5 mg/ml collagen using a polydimethylsiloxane microstamp as previously described (Moulder et al., 2007). Cortical astrocytes from postnatal d 4 male and female rat pups were seeded on these islands in Eagle's medium (Life Technologies) supplemented with 5% heat-inactivated horse serum, 5% fetal bovine serum, 17 mM D-glucose, 400 μM glutamine, 50 U/ml penicillin, and 50 $\mu\text{g}/\text{ml}$ streptomycin. Glial preplates were maintained in a humidified and atmospherically controlled incubator (5% $\text{CO}_2/95\%$ air at 37°C) prior to neuronal plating. Astrocyte-poor cultures were prepared 7-10 d after plating by fixation with 4% paraformaldehyde/0.2% glutaraldehyde in phosphate-buffered saline (PBS) at room temperature (RT) for 5 min prior to at least 3 washes in PBS. Alternatively, 70% ethanol at -20°C was applied for 30 min prior to PBS rinses. Although most experiments used aldehyde fixation, both astrocyte fixation protocols produced the finding shown in Figure 1B. For mass cultures used in Figure 5, a confluent layer of polylysine/laminin was applied to the culture dish prior to neuronal plating.

Hippocampal neurons were dispersed on fixed or live astrocyte beds for microcultures while astrocytes and neurons were co-plated for mass cultures as previously described (Mennerick et al., 1995). Briefly, male and female postnatal d 0-3

Sprague Dawley rat hippocampi were dissected, incubated with 1 mg/ml papain, and mechanically dissociated. To encourage the formation of autaptic (solitary) neurons in microcultures, cells were plated at low density (~ 100 cells/mm²). For mass cultures, cells were plated at high density (~ 650 cells/mm²) to encourage neuron-astrocyte co-cultures to form. After 3-4 d, 6.7 μ M cytosine arabinoside was added to inhibit cell division. One half of the culture medium was exchanged with Neurobasal medium (Life Technologies) plus B27 supplement at DIV 4-5. Conditioned media were from astrocyte cultures or from neuron-astrocyte co-cultures; similar results were obtained with both. Non-conditioned medium was fresh Neurobasal without supplements. Unless otherwise stated, all experiments were performed at 10-14 DIV, and controls were sibling cultures used the same day.

Electrophysiology:

Whole-cell voltage-clamp recordings were performed on autaptic neurons in microisland culture unless otherwise stated. Data were collected using pClamp 9 software with a Multiclamp 700B or Axopatch 200B amplifier and Digidata1322A data acquisition board (Molecular Devices). Recording saline typically contained 138 mM NaCl, 4 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, 10 mM HEPES, and 25 μ M D-APV (Tocris Bioscience) at a pH of 7.25. For experiments requiring NMDA receptor activation, 10 μ M glycine (Tocris Bioscience) was added to the saline solution and MgCl₂ was excluded. Pipette solution contained 140 mM potassium gluconate, 4 mM NaCl, 0.5 mM, 0.5 mM CaCl₂, 5 mM EGTA, and 10 mM HEPES at a pH of 7.25. For experiments in which both excitatory and inhibitory postsynaptic currents (EPSCs

and IPSCs) were collected during the same recording session from autaptic neurons, potassium gluconate was replaced with 140 mM KCl. For network activity experiments measuring spontaneous EPSCs and IPSCs in mass cultures, 130 mM cesium methanesulfonate replaced potassium gluconate.

Recordings were performed at room temperature. Electrode pipettes were pulled from borosilicate glass (World Precision Instruments) and had 3-6 M Ω resistance. Access resistance was compensated 85-100%, and membrane potential was typically held at -70 mV. For network activity experiments in mass cultures, neurons were voltage-clamped at -30 mV or -35 mV, midway between IPSC and EPSC reversal potentials, without compensation to measure spontaneous network activity due to excitation and inhibition simultaneously. Input resistance (astrocyte-rich: 189.3 ± 39.3 M Ω ; astrocyte-poor: 212.4 ± 76.3 M Ω ; $n=11-12$ neurons; $p=0.77$, Student's unpaired t test) and cell capacitance (astrocyte-rich: 73.3 ± 7.3 pF; astrocyte-poor: 94.0 ± 10.3 pF; $n=14-16$ neurons; $p=0.11$, Student's unpaired t test) were similar in neurons from astrocyte-poor cultures compared with sibling astrocyte-rich cultures. Autaptic EPSCs and IPSCs were evoked by a 1.5 ms pulse depolarization to 0 mV. Paired-pulse responses were obtained by evoking two such depolarizing pulses 50 ms apart. Signals were sampled at 10 kHz and low-pass filtered at 4 kHz, except for miniature EPSCs collected at 5 kHz and filtered at 1 kHz and spontaneous EPSCs and IPSCs collected at 5 kHz and filtered at 2 kHz. Autaptic EPSCs were recorded up to 1 h after switch to recording saline. When solutions were applied acutely to neurons, a multibarrel perfusion system was used with a common port placed within 0.5 mm of the neuron; solution exchange times were ~ 100 ms.

Glutamate receptor blockers (50 μ M D-APV and 1 μ M NBQX) were present

during all depolarization challenges (30 mM KCl for depolarization or NaCl as a non-depolarized osmotic control) to prevent toxicity and NMDA receptor-dependent plasticity. Control treatments for all other experiments consisted of vehicle treatment unless otherwise specified.

Immunostaining and Microscopy:

FM1-43 dye labeling was performed as previously described (Moulder et al., 2010). Briefly, microisland cultures were treated for 2 min with 10 μ M fixable FM1-43 (FM1-43FX; Life Technologies) in recording saline supplemented with 45 mM KCl and 1 μ M NBQX. Immediately following, the culture dishes were briefly washed (5s) in saline supplemented with 500 μ M Advasep-7 (CyDex) and 1 μ M NBQX. Cells rested during 10 min of washes with saline plus 1 μ M NBQX and were then fixed for 10 min.

Fixation prior to immunostaining was 4% paraformaldehyde at RT, 4% paraformaldehyde/0.2% glutaraldehyde at RT, 4% paraformaldehyde/0.02% glutaraldehyde at RT, or 100% methanol at -20 °C. All culture dishes were washed with PBS at RT followed by 2% normal goat serum in PBS blocking solution plus 0.1% or 0.04% Triton X-100. Primary antibodies were vesicular glutamate transporter 1 (vGluT-1; 1:2000; Millipore), MAP2 (1:2000; Millipore), phospho-synapsin (1:400; Millipore), synapsin (1:2000; Millipore), phospho-cAMP response element binding protein (phospho-CREB; 1:400; Millipore), γ -aminobutyric acid (GABA; 1:500), and phospho-dynamin 1 (1:100; Santa Cruz Biotechnology). Primary antibodies were applied for 2-3 h at RT before PBS wash, 30-40 min secondary antibody incubation with Alexa Fluor conjugates (Life Technologies), and glass coverslipping with Fluoromount G (Southern

Biotechnology Associates).

FM1-43FX and immunostaining images were acquired on an inverted Eclipse TE2000-S microscope with a 60X objective (1.4 numerical aperture) using a C1 scanning confocal laser (488, 543, and/or 633 nm) and EZ-C1 software (Nikon). Alternating laser lines were used to obtain z-stack images while all gain and acquisition settings were held constant within a given experiment. Two-dimensional projected images were created and analyzed using MetaMorph 7 software (Universal Imaging).

Live cell images were obtained on an Eclipse TE2000-S inverted microscope (Nikon) with a 40X objective (0.6 numerical aperture; Nikon) and a metal halide lamp. Images were acquired with MetaMorph 7 software and a cooled 12-bit CCD camera (Photometrics).

Data Analysis:

Data were analyzed and graphed using MetaMorph 7, Clampfit 9 (Molecular Devices), Mini Analysis 6 (Synaptosoft Inc.), Excel 2007 (Microsoft), and/or SigmaPlot 10 (Systat) software. Unless otherwise stated, data are displayed as mean \pm SEM, and Student's unpaired *t* test was used to compare 2 groups while a Bonferroni correction was applied when experiments contained more than 2 groups. A corrected *p* value of < 0.05 was required to reach significance. The reported *n* refers to the sample size of each group within an experiment.

At least 3 EPSC amplitudes were averaged for each autaptic neuron. For each recording day, responses from experimental treatments were normalized to the average response in the control-treated sibling culture. For dual-component autaptic EPSCs, peak

EPSC amplitude estimated the AMPA receptor component while the average current 80-100 ms following the peak estimated the NMDA receptor component (Hestrin et al., 1990). Miniature EPSCs were elicited by perfusion of 100 mM sucrose and analyzed using Mini Analysis software with manual confirmation. The first 57 miniature EPSC inter-event interval and amplitude values were used for Kolmogorov-Smirnov tests while values for all miniature EPSCs from a given neuron were averaged for Mann-Whitney U tests.

At least 10 fields per culture dish were used to calculate the percentage of live (calcein-positive) islands. For the percentage of FM1-43-positive vGluT-1 synapses, regions of interest were manually drawn around 10 vGluT-1 puncta per field for 5 fields per culture dish. As previously described (Crawford et al., 2011), FM1-43 images were thresholded before analyzing vGluT-1-defined regions, and synapses were considered active if at least 10 pixels reached threshold. Similarly, 10 vGluT-1-defined regions were manually drawn per field before analyzing synapsin and phospho-synapsin images. The number and intensity of vGluT-1-positive synapses in autaptic neurons was quantified via automatic detection in thresholded images using MetaMorph 7 software. Most immunostaining experiments used glutamatergic autaptic neurons, identified by vGluT-1 immunoreactivity. Glutamatergic neurons on multi-cell islands were analyzed for nuclear phospho-CREB experiments, however, and antibody incompatibility precluded double labeling in phospho-dynamin 1 experiments. For display in figures, all images within a panel were pseudocolored and adjusted for brightness and contrast equivalently.

Materials:

Rp-cAMPS and 2-chloro-N⁶-cyclopentyladenosine (CCPA) were obtained from Tocris Bioscience, kainic acid was purchased from BioVectra, calcein-AM was purchased from AnaSpec, Inc., human recombinant thrombospondin 1 (TSP1) was obtained from Haematologic Technologies, Inc., and tumor necrosis factor alpha (TNF α) was obtained from EMD4 Biosciences. All other materials were obtained from Sigma-Aldrich unless otherwise specified.

Results

We reduced glial signaling in rat hippocampal cultures by growing neurons on fixed or live astrocyte beds (Fig. 1A). This strategy permitted identical culture conditions for astrocyte-rich and astrocyte-poor cultures, except for the number of viable astrocytes. It also allowed control neurons the full benefit of local and global astrocytic cues, unlike preparations where control neurons only receive soluble astrocytic signals (Kaech and Banker, 2006). EPSCs from day *in vitro* (DIV) 10-14 autaptic neurons in astrocyte-rich microcultures were persistently depressed with as little as 60 min depolarization, as assessed up to 1 h following the removal of depolarization (Fig. 1B and 1C), while 30 minutes of depolarization yielded insignificant muting ($p=0.45$). We have shown previously that this depolarization-induced depression is selective for glutamate synapses and arises from muting in the absence of synapse loss or postsynaptic receptor changes (Moulder et al., 2004; Moulder et al., 2006). By contrast, neurons in astrocyte-poor cultures exhibited no EPSC depression with up to 4 h depolarization, which produced strong muting in astrocyte-rich cultures (Fig. 1B and 1C). Muting, therefore, is impaired in astrocyte-poor cultures.

To evaluate whether astrocyte-deprived neurons simply failed to depolarize properly, and to test an alternative method of muting induction, we incubated cells in $G_{i/o}$ -coupled receptor agonists CCPA (an A1 adenosine receptor agonist) and baclofen (a $GABA_B$ receptor agonist) or in cAMP-activated protein kinase A (PKA) antagonist Rp-cAMPS, both of which have been shown to induce presynaptic muting (Moulder et al., 2008; Crawford et al., 2011). These agents depressed EPSCs in astrocyte-rich cultures, as expected, but significantly increased EPSC amplitudes in astrocyte-poor cultures (Fig.

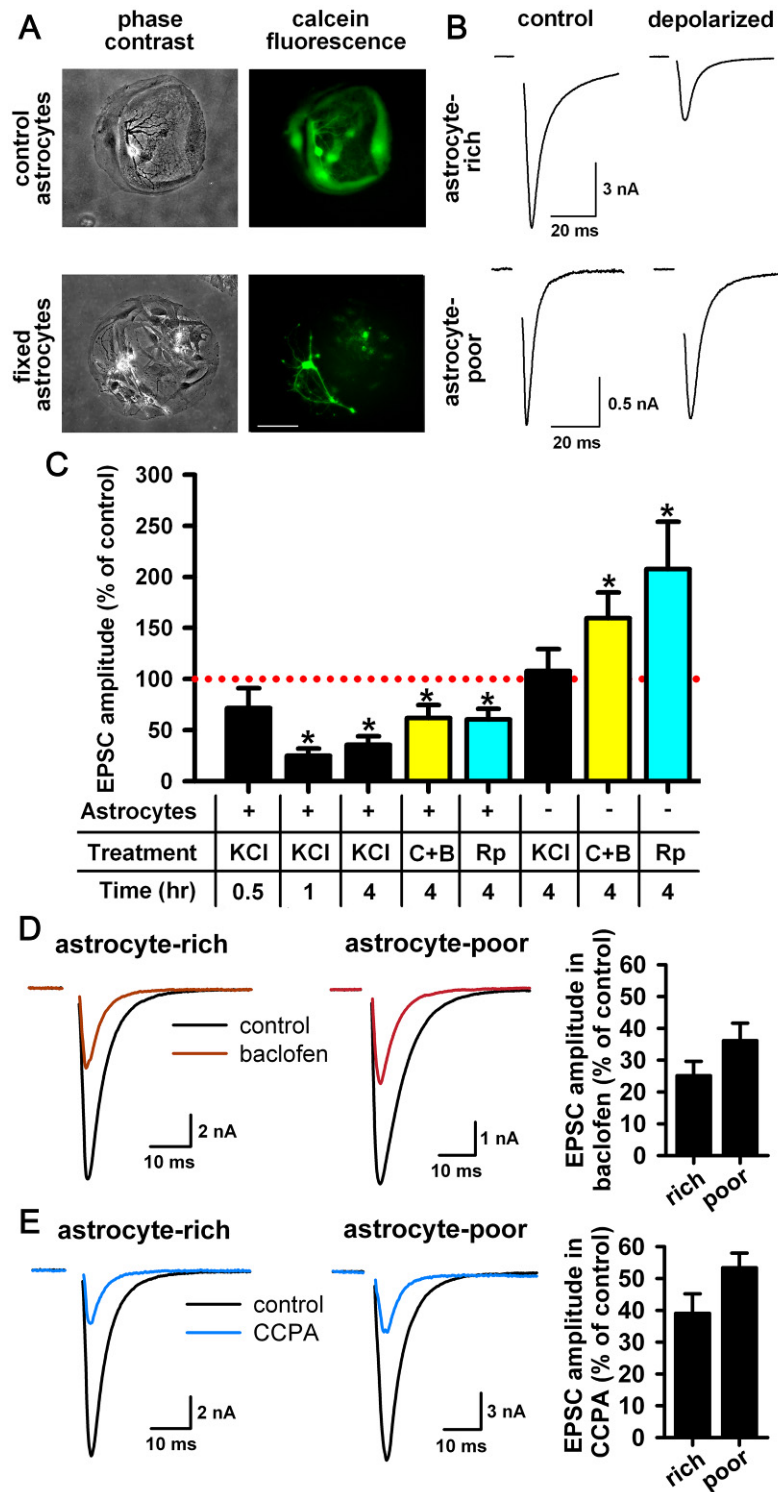


Figure 1. Astrocyte deprivation impairs presynaptic muting but not G-protein activation. **A.** Phase-contrast and fluorescence images of autaptic neurons on viable (control) or non-viable (4% paraformaldehyde/0.2% glutaraldehyde-fixed) glial “islands” acquired 30 min after 5 μ g/ml calcein-AM treatment. Fewer islands contained live (calcein-positive) astrocytes in fixed cultures (control: $98.8 \pm 0.9\%$ live islands; fixed:

17.6 ± 2.8% live islands; $n=6$ dishes; $p=7.9 \times 10^{-11}$, Student's unpaired t test). Scale bar represents 40 μm . **B.** Representative autaptic EPSCs from astrocyte-rich or astrocyte-poor microcultures recorded in normal saline up to 1 h after 4 h control (30 mM NaCl) or depolarization (30 mM KCl). **C.** Summary EPSCs from DIV 10-14 astrocyte-rich (+) or astrocyte-poor (-) autaptic neurons after indicated treatment times with 30 mM KCl (black bars), 10 nM CCPA (A1 adenosine receptor agonist) plus 50 μM baclofen (GABA_B receptor agonist) (C+B; yellow bars), or 50 μM Rp-cAMPS (Rp; cyan bars). EPSCs were normalized to the average EPSCs from control-treated sibling neurons for each recording day ($n=14-22$ neurons; $*p<0.05$ vs. treatment controls, Student's unpaired t test). **D.** Left: EPSCs from astrocyte-rich or astrocyte-poor cultures after acute perfusion with recording saline (control) or 20 μM baclofen. Right: summary of EPSC depression from acute perfusion of baclofen ($n=11$ neurons; $p=0.14$, Student's unpaired t test). **E.** Left: EPSCs from astrocyte-rich or astrocyte-poor cultures after acute perfusion with recording saline (control) or 10 nM CCPA. Right: summary of EPSC depression from acute perfusion of CCPA ($n=11$ neurons; $p=0.08$, Student's unpaired t test). The same neurons were used for panel D and panel E experiments, using an interleaved protocol between saline and agonist-containing solutions.

1C). This unexpected reversal of EPSC depression indicated an abnormality in muting induction. Meanwhile, canonical acute presynaptic depression, mediated by G $\beta\gamma$ subunit signaling (Brown and Sihra, 2008), did not differ in astrocyte-poor cultures (Fig. 1D and 1E). Therefore, the muting deficit in astrocyte-deprived neurons is likely downstream of depolarization and G $\alpha_{i/o}$ subunit-mediated cAMP signaling.

Basal EPSC amplitude was smaller in astrocyte-deprived cultures than in astrocyte-rich cultures (astrocyte-rich: -18.3 ± 5.7 nA; astrocyte-poor: -5.0 ± 1.4 nA; $n=15-16$; $p=0.037$, Student's unpaired t test); however, IPSCs were similar (astrocyte-rich: -7.6 ± 2.5 nA; astrocyte-poor: -8.7 ± 1.9 nA; $n=14$ neurons; $p=0.74$, Student's unpaired t test), suggesting that the basal deficit in amplitude in astrocyte-poor cultures localized specifically to glutamatergic synapses. The decreased amplitude was not explained by differences in local astrocytic signaling because neurons in astrocyte-deprived cultures on an island containing one of the few contaminating live astrocytes did not have larger EPSCs than neurons associated with non-viable islands, as assessed by a vital stain (Fig. 2A). A global deficit in astrocyte-poor cultures, therefore, likely explains the basal EPSC depression.

Decreased functionally active presynaptic terminals did not explain the basal EPSC depression or the deficient stimulus-induced muting in astrocyte-poor cultures. The percentage of basally mute terminals, as measured via absence of stimulus-induced FM1-43FX dye labeling of vesicles at vGluT-1-immunoreactive synapses, was similar in astrocyte-rich and astrocyte-poor cultures (Fig. 2B). Furthermore, neither the number nor the intensity of vGluT-1 puncta per cell differed, suggesting normal synapse and vesicle numbers (Fig. 2C). Paired-pulse EPSC depression was also normal, suggesting similar

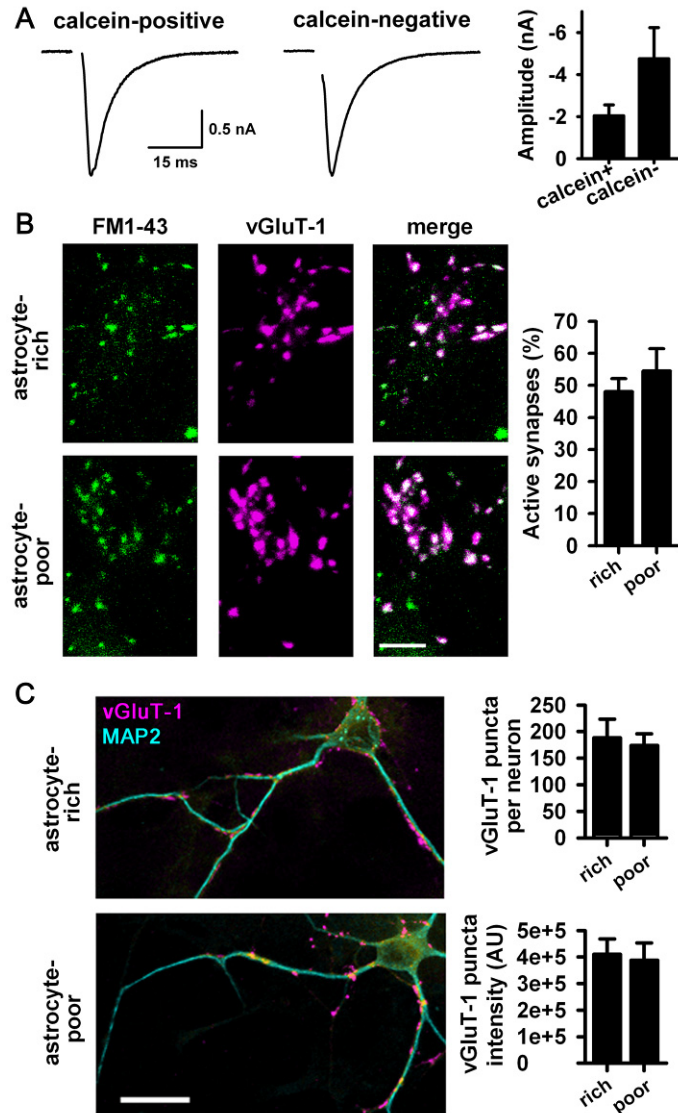


Figure 2. Astrocyte deprivation does not impair basal presynaptic function. **A.** EPSCs from autaptic neurons in astrocyte-poor cultures from islands with residual live astrocytes (calcein-positive) or no live astrocytes (calcein-negative). The few local astrocytes in astrocyte-poor cultures did not significantly affect EPSC amplitude ($n=13-16$ neurons; $p=0.13$, Student's unpaired t test). **B.** Left: images of $10 \mu\text{M}$ FM1-43FX labeling of presynaptic terminals (green) after 2 min 45 mM KCl and subsequent vGluT-1 immunostaining (magenta) in autaptic neurons from astrocyte-rich and astrocyte-poor cultures (30 min -20°C 70% EtOH-fixed astrocytes). Scale bar represents $5 \mu\text{m}$. Right: quantification of the percentage of active synapses, as defined by FM1-43 co-localization with vGluT-1 ($n=8$ dishes; $p=0.44$, Student's unpaired t test). **C.** Left: vGluT-1 (magenta) and MAP2 (cyan) immunostaining in autaptic neurons showing similar density and intensity of presynaptic terminals. Scale bar represents $20 \mu\text{m}$. Right: summary of vGluT-1 puncta per autaptic neuron ($n=35$ neurons; $p=0.74$, Student's unpaired t test) and vGluT-1 integrated fluorescence intensity ($n=35$ neurons; $p=0.80$, Student's unpaired t test).

vesicle release probability (astrocyte-rich: $5.1 \pm 9.2\%$; astrocyte-poor: $18.2 \pm 8.6\%$; $n=15-16$; $p=0.3$, Student's unpaired t test). No deficits in presynaptic function, therefore, were detected with reduced astrocytic signaling.

A postsynaptic deficit, rather, appeared to explain the basal EPSC depression in astrocyte-poor cultures. AMPA receptor and NMDA receptor components of EPSCs were reduced in parallel (Fig. 3A), and this parallel depression was associated with decreased responsiveness to exogenously applied selective agonists for both receptor types (Fig. 3B). Additionally, miniature EPSC (mEPSC) amplitude was reduced (Fig. 3C), further suggesting that postsynaptic receptor levels are decreased in astrocyte-poor cultures. Frequency of mEPSCs also trended toward reduction (Fig. 3C), but it was unclear whether this resulted from an undetected presynaptic change or whether the smallest mEPSCs fell below the detection threshold. To ask whether the decreased basal EPSC amplitude contributed to the muting deficit in astrocyte-poor cultures, we attempted to induce muting in young (DIV 7-8), astrocyte-rich cultures. Despite smaller basal EPSC amplitudes similar to those found in DIV 10-14 astrocyte-poor cultures (-3.75 ± 0.95 nA; $n=17$), these younger neurons in astrocyte-rich cultures expressed muting in response to a 4 h depolarization challenge (Fig. 4A and 4E). Conversely, older (DIV 21), astrocyte-poor cultures with larger basal EPSC amplitudes (-10.27 ± 4.02 nA; $n=12$) did not exhibit muting (Fig. 4E). Together with prior published studies suggesting that postsynaptic glutamate receptor function is not required for muting induction (Moulder et al., 2004; Moulder et al., 2006), these data suggest that the postsynaptic changes caused by astrocyte deficiency do not explain the muting deficits. Because glial modulation of glutamate receptor levels and function has been studied previously (Beattie et al., 2002;

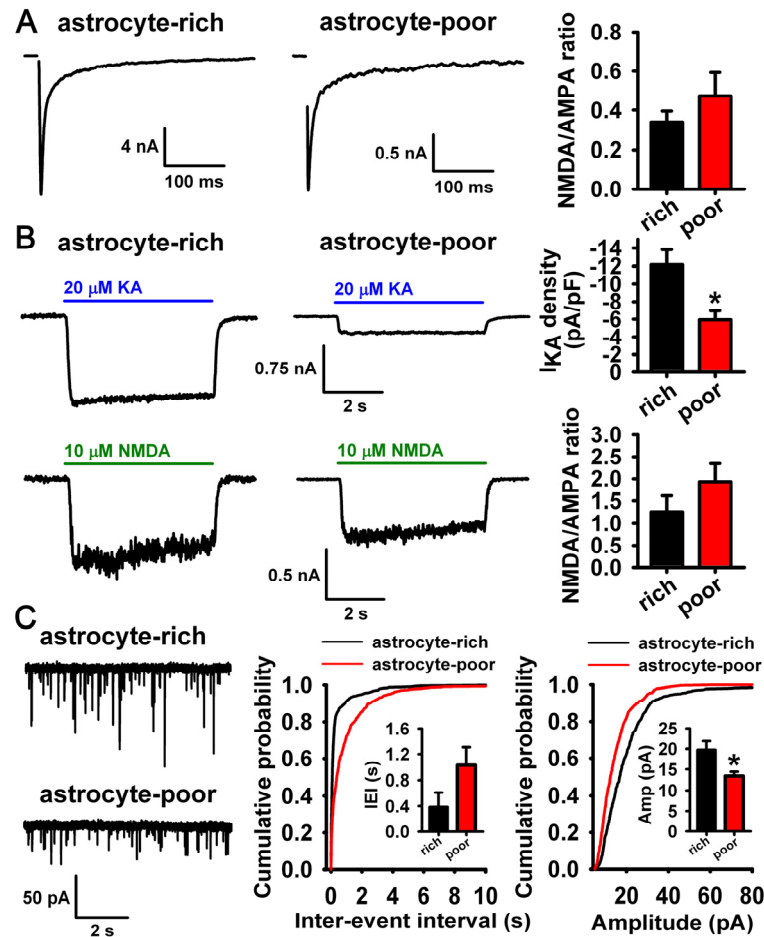


Figure 3. Glutamate receptor levels are decreased in astrocyte-poor cultures. A. Left: Representative dual-component (NMDA and AMPA receptor-mediated) EPSCs elicited in autaptic neurons from astrocyte-rich or astrocyte-poor cultures. Right: quantification of the ratio of the NMDA to AMPA receptor component of EPSCs ($n=14-15$ neurons; $p=0.34$, Student's unpaired t test). **B.** Top traces: currents from autaptic neurons in astrocyte-rich or astrocyte-poor cultures in response to acute kainic acid (KA) perfusion, a non-desensitizing AMPA receptor agonist. Bottom traces: currents from autaptic neurons in astrocyte-rich or astrocyte-poor cultures in response to acute NMDA perfusion to selectively activate NMDA receptors. Right: quantification of the current density in response to KA ($n=14-15$ neurons; $p=0.004$, Student's unpaired t test) or of the ratio of the NMDA response to the KA response ($n=14-15$ neurons; $p=0.16$, Student's unpaired t test). Although the overall response to receptor activation was depressed in astrocyte-poor cultures, NMDA to AMPA receptor current ratios did not differ significantly. **C.** Left: representative miniature EPSCs from astrocyte-rich or astrocyte-poor cultures elicited by acute perfusion of 100 mM sucrose. Right: cumulative probability plots of inter-event interval ($p<0.001$ with $D=0.3860$) and amplitude ($p<0.001$ with $D=0.2175$) of miniature EPSCs from 10 astrocyte-rich and 10 astrocyte-poor neurons (Kolmogorov-Smirnov test). Insets: average \pm SEM miniature EPSC inter-event interval ($n=10$ neurons; $p>0.05$, Mann-Whitney U test) and amplitude ($n=10$ neurons; $p<0.01$, Mann-Whitney U test).

Stellwagen and Malenka, 2006; Perea et al., 2009; Sullivan et al., 2011), we did not further characterize the mechanisms responsible for this postsynaptic deficit in astrocyte-poor cultures.

Astrocytes could directly instruct muting during depolarization (e.g. through gliotransmission), or they could permit development of muting competence (e.g. through global cues). In support of the latter, conditioned medium from mature, live-astrocyte cultures added to astrocyte-poor cultures at DIV 7 rescued muting, even when conditioned medium was replaced with fresh medium during the depolarization challenge (Fig. 4B and 4E). Conditioned medium was ineffective if applied only during the muting induction protocol (Fig. 4C and 4E). Thus, glial factors are not released instructively during muting induction and are, instead, released permissively during synapse development. Because older (DIV 21), astrocyte-poor cultures failed to express muting (Fig. 4E), it is unlikely that muting competence is delayed in the absence of astrocytes. Rather, it appears that astrocytes provide a fundamental developmental cue that permits muting competence.

We hypothesized that astrocytes release a permissive substance for muting development rather than remove a non-permissive factor. We tested effects of adding human thrombospondin 1 (TSP1; 5 μ g/ml) to astrocyte-poor cultures because astrocyte-derived TSPs foster presynaptic maturation via binding to the neuronal α 2 δ -1 subunit of voltage-gated calcium channels or to neuroligin 1 (Eroglu et al., 2009; Xu et al., 2010). Incubation in human TSP1 starting at DIV 7 rescued presynaptic muting competence in astrocyte-poor cultures (Fig. 4D and 4E). In contrast, tumor necrosis factor alpha (TNF α ; 1 ng/ml), an astrocyte-derived factor involved in postsynaptic plasticity (Beattie et al.,

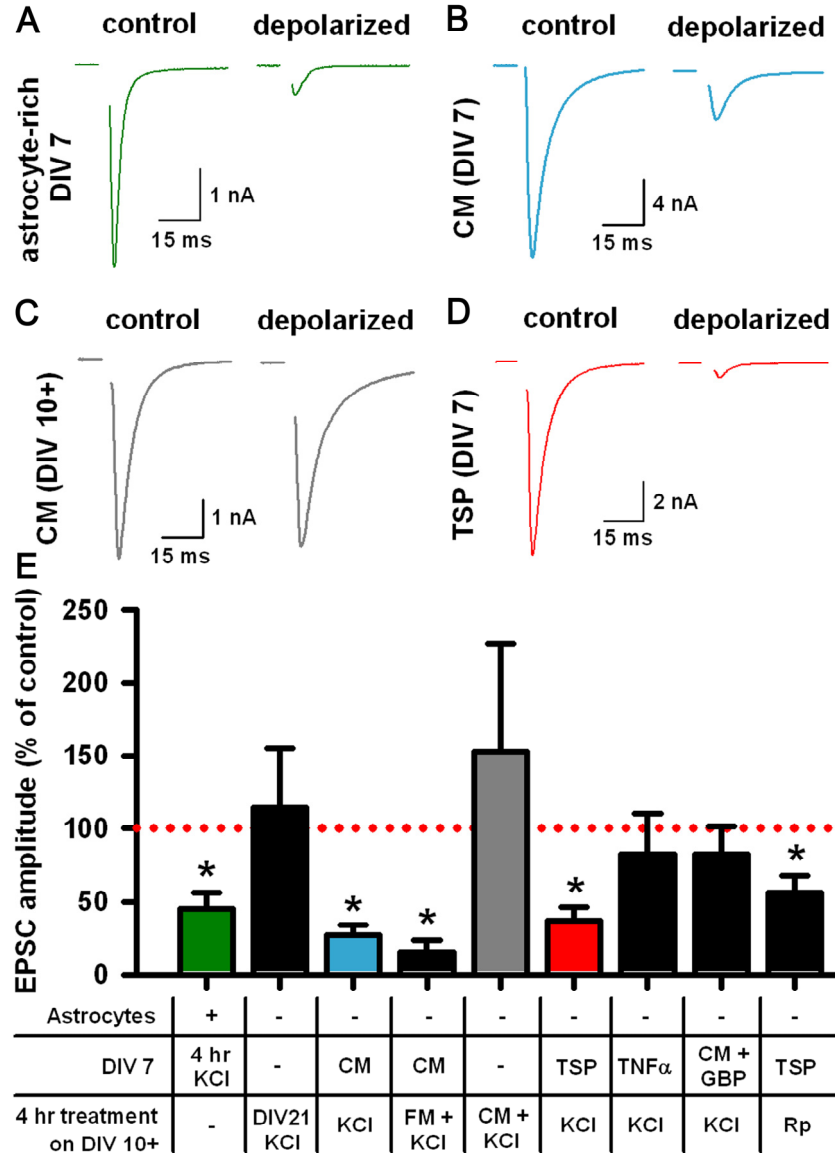


Figure 4. Soluble, astrocyte-derived thrombospondin rescues presynaptic muting competence. A-D. Representative DIV 7-12 autaptic EPSCs after 4 h control (30 mM NaCl) or depolarization (30 mM KCl). Cultures were (A) astrocyte-rich and challenged at DIV 7 without additional treatments, (B) astrocyte-poor and treated with astrocyte-conditioned medium (CM) at DIV 7 and challenged at DIV 10-12, (C) astrocyte-poor and challenged at DIV 10-12 in CM, or (D) astrocyte-poor and treated with 5 μ g/ml human thrombospondin 1 (TSP) at DIV 7 and challenged at DIV 10-12. E. Summary of EPSCs after 4 h 30 mM KCl or 50 μ M Rp-cAMPS (Rp) with indicated pretreatments and/or co-treatments ($n=12-20$ neurons; $*p<0.05$ vs. treatment controls, Student's unpaired t test). EPSCs were normalized as in Figure 1C. Abbreviations: astrocyte-rich cultures (+), astrocyte-poor cultures (-), 1 ng/ml tumor necrosis factor alpha (TNF α), 32 μ M gabapentin (GBP), and fresh, non-conditioned medium (FM).

2002; Stellwagen and Malenka, 2006), did not rescue muting in astrocyte-poor cultures (Fig. 4E). These data suggest that TSP1 is capable of promoting muting competence but do not clarify whether TSPs are the endogenous astrocytic factors responsible.

To test for the receptor through which TSP1 promotes muting, we blocked TSP binding to the candidate receptor $\alpha 2\delta$ -1 using gabapentin, a clinically used drug for the treatment of neuropathic pain and epilepsy. Gabapentin interferes with TSP binding to $\alpha 2\delta$ -1 and prevents TSP's synaptogenic effects in retinal ganglion neurons (Gee et al., 1996; Eroglu et al., 2009). In astrocyte-poor cultures, gabapentin (32 μ M) abolished conditioned medium rescue of depolarization-induced muting (Fig. 4E), suggesting that TSPs are likely the astrocyte-derived regulators of muting competence. We also evaluated the effect of gabapentin in live-astrocyte cultures, where ongoing production of astrocytic TSPs was an additional consideration. In these cultures, a single treatment with gabapentin at DIV 7 was ineffective in preventing silencing, as assessed at DIV 10-12 (Fig. 5A and 5C); however, 3-5 d maintenance of gabapentin by partial medium exchange in astrocyte-rich cultures eliminated depolarization-induced muting (Fig. 5B and 5C). These results suggest that gabapentin also blocks endogenously-released factors important for muting when the ongoing production of the factors is taken into account.

To test whether use of this drug to block endogenous TSP could have implications for neural network function, we measured spontaneous network activity after muting induction in control- and gabapentin-treated cultures. These astrocyte-rich, conventional mass cultures exhibited decreased spontaneous activity after muting induction (Fig. 5D and 5E), consistent with an adaptive role for muting (Hogins et al., 2011; Crawford and Mennerick, 2012). In gabapentin-treated cultures, however, a non-significant increase in

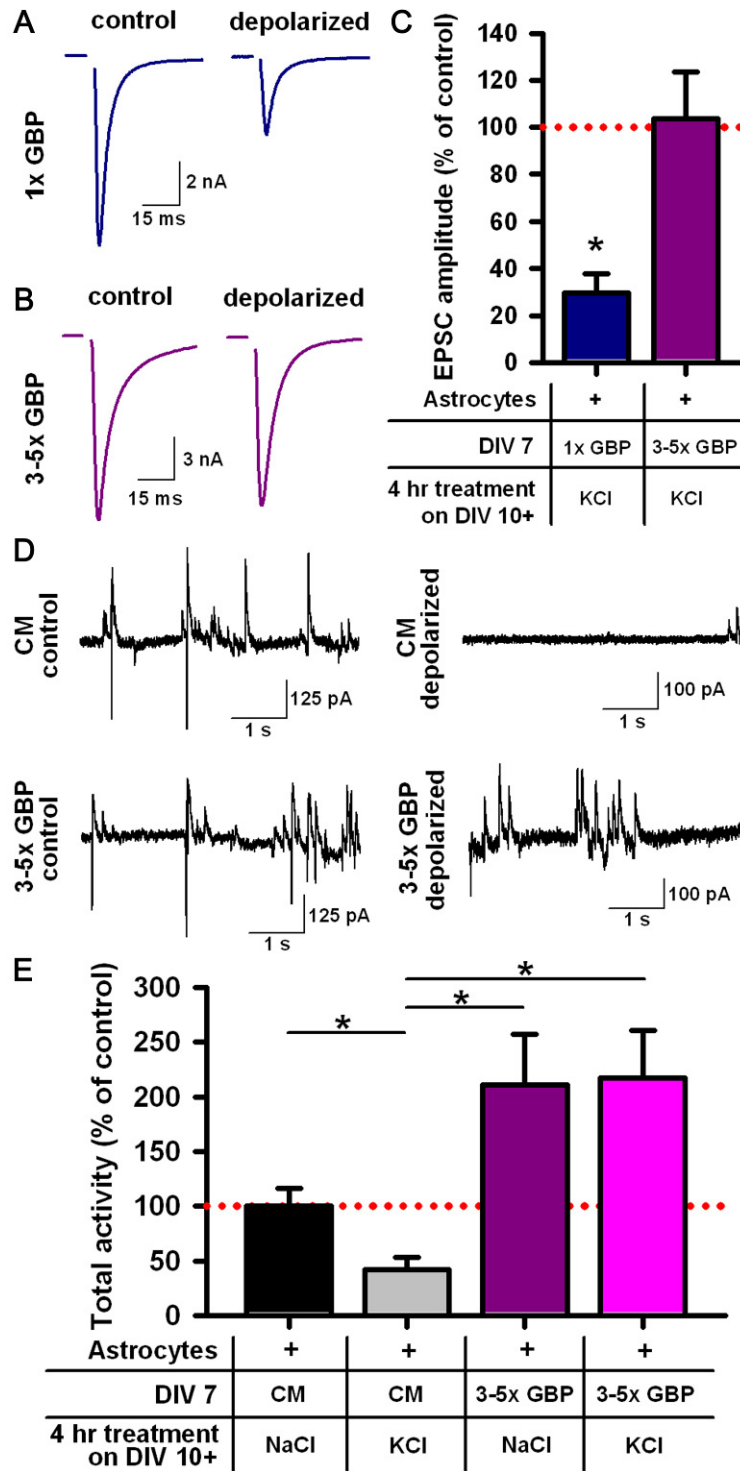


Figure 5. Gabapentin prevents the development of muting competence. A. Representative DIV 10-12 autaptic EPSCs after 4 h control (30 mM NaCl) or depolarized (30 mM KCl). Astrocyte-rich cultures were treated with 32 μ M gabapentin at DIV 7 (1x GBP). **B.** DIV 10-12 autaptic EPSCs after 4 h control or depolarized. Astrocyte-rich cultures were treated with 32 μ M gabapentin at DIV 7 followed by half-media switches of fresh 32 μ M gabapentin in astrocyte-conditioned medium every day (3-5x GBP). **C.**

Summary of DIV 10-12 autaptic EPSCs after 4 h 30 mM KCl with indicated pretreatments ($n=12-14$ neurons; $*p<0.05$ vs. treatment controls, Student's unpaired t test). EPSCs were normalized as in Figure 1C. **D.** Spontaneous activity in DIV 11-13 mass cultures after 4 h control (30 mM NaCl) or depolarized (30 mM KCl). Cultures were treated at DIV 7 with or without 32 μ M gabapentin followed by daily half media switches with astrocyte-conditioned medium (CM) or CM plus 32 μ M gabapentin (3-5x GBP). EPSCs and IPSCs were measured simultaneously at -35 mV. **E.** Summary of total spontaneous synaptic activity (EPSC + IPSC points) in DIV 11-13 mass cultures, normalized to control (CM, NaCl) for each recording day ($n=23$ neurons; $*p<0.05$, Bonferroni corrected).

network activity was observed (Bonferroni corrected $p=0.16$), but no change in network activity occurred after the muting induction protocol (Fig. 5D and 5E), suggesting that muting was not induced. Together, these results argue that TSP binding to $\alpha 2\delta$ -1 fosters development of presynaptic muting competence.

Although the signaling pathways downstream of $\alpha 2\delta$ -1 are unclear, we hypothesized that PKA signaling, which regulates muting (Moulder et al., 2008), is altered in astrocyte-deprived neurons. This hypothesis was based in part on the observations that both $G_{i/o}$ -coupled receptor agonist-induced muting and Rp-cAMPS-induced muting were dysfunctional (Fig. 1C). To test whether PKA activity was normal in astrocyte-poor cultures, we measured phosphorylation levels of PKA substrates in neurons. We found that phosphorylation levels of the presynaptic protein synapsin at its PKA phosphorylation site were increased in astrocyte-poor cultures despite unchanged levels of total synapsin (Fig. 6). Phosphorylation of cAMP response element-binding protein, another PKA substrate, was also abnormally high in neuronal nuclei from astrocyte-deprived cultures (Fig. 7A). In contrast, phosphorylation of dynamin 1, a PKC substrate, was unchanged in astrocyte-poor cultures (Fig. 7B), suggesting that the over-phosphorylation phenotype was restricted to PKA phosphorylation sites. TSP1 reversed abnormal presynaptic phospho-synapsin (Fig. 6A and 6B), so TSP appears to restore normal PKA targeting and/or activity. Furthermore, TSP1 incubation reinstated normal Rp-cAMPS-induced persistent EPSC depression in astrocyte-poor cultures (Fig. 4E). Together, these results suggest that TSP promotes muting competence via normalization of signaling downstream of cAMP pathways.

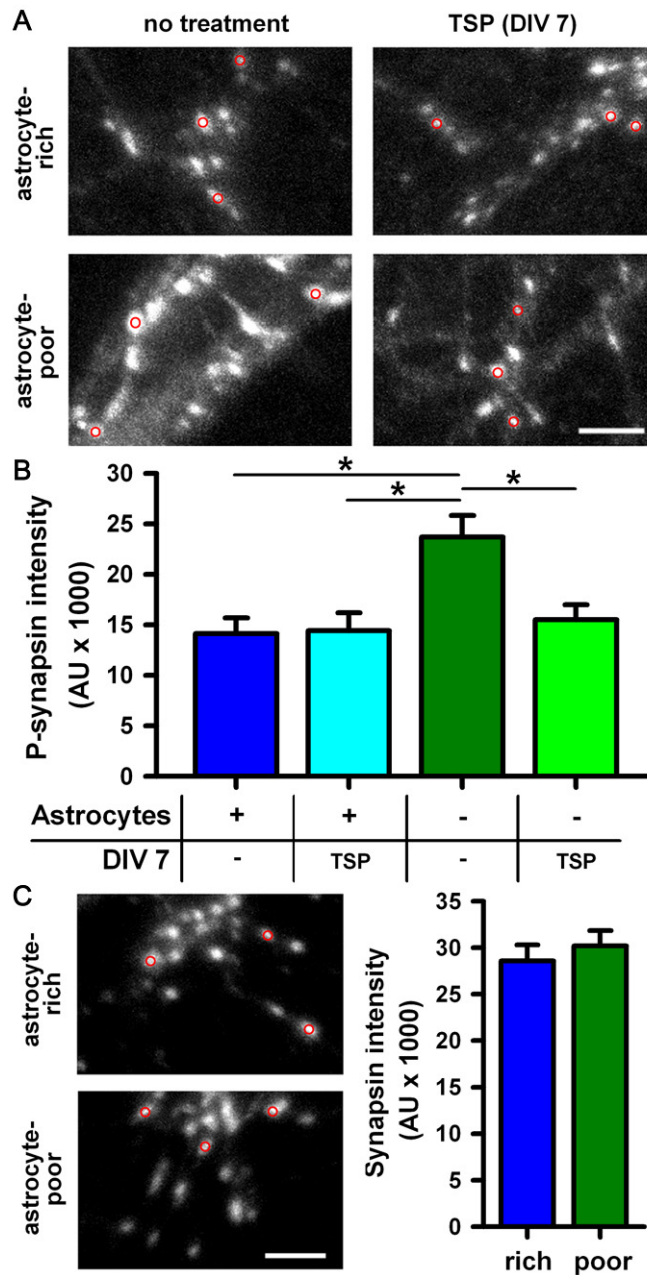


Figure 6. PKA-dependent hyper-phosphorylation is normalized by thrombospondin.

A. Phospho-synapsin (PKA phosphorylation site) immunostaining in DIV 10-12 autaptic neurons from astrocyte-rich or astrocyte-poor cultures with or without 5 $\mu\text{g/ml}$ thrombospondin (TSP) treatment at DIV 7. Red circles are representative regions of interest defined by vGluT-1 immunoreactivity (not shown). Scale bar represents 5 μm . **B.** Summary of background-subtracted phospho-synapsin at vGluT-1-positive synapses in DIV 10-12 autaptic neurons ($n=35$ neurons; $*p<0.05$, Bonferroni corrected). **C.** Left: synapsin immunostaining in autaptic neurons from astrocyte-rich or astrocyte-poor cultures. Right: summary of background-subtracted synapsin immunoreactivity ($n=35$ neurons; $p=0.51$, Student's unpaired t test) at vGluT-1-defined synapses (red circles are representative). Scale bar represents 5 μm .

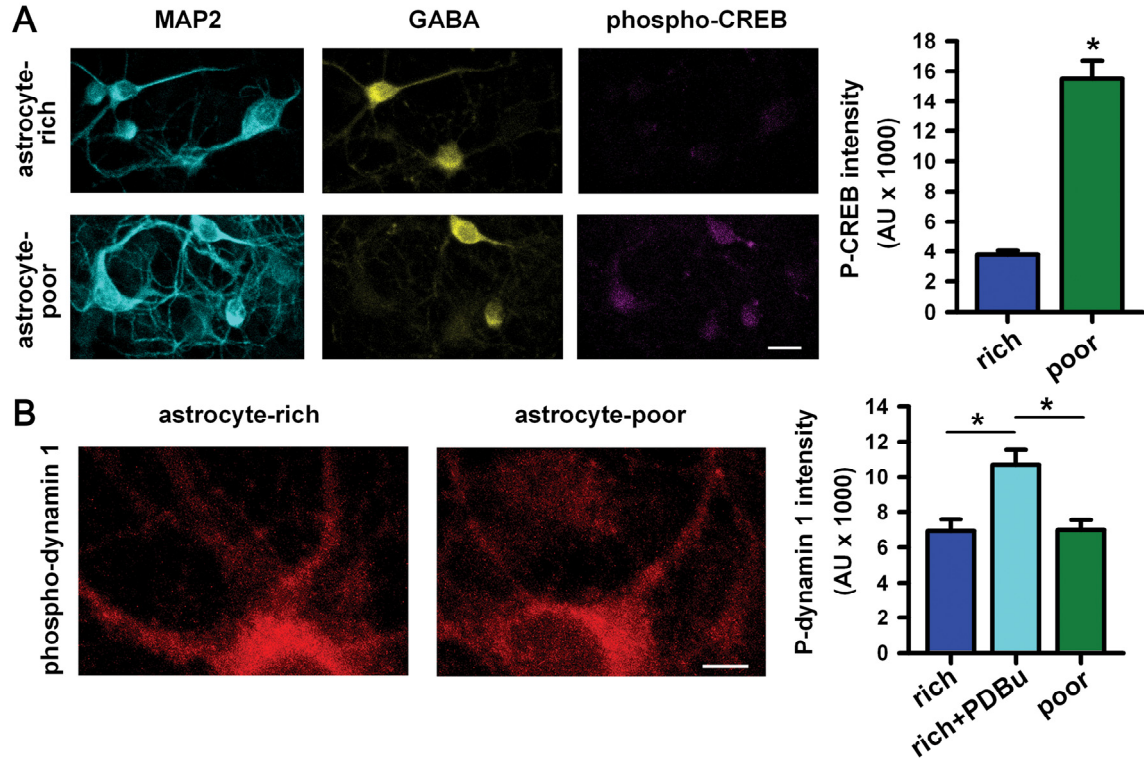


Figure 7. A PKA target, but not a non-PKA target, is hyper-phosphorylated in astrocyte-poor cultures. **A.** Left: MAP2, GABA, and phospho-CREB (PKA phosphorylation site) immunoreactivity in neurons on multi-cell islands from astrocyte-rich or astrocyte-poor cultures. Scale bar represents 20 μm . Right: quantification of background-subtracted phospho-CREB intensity from GABA-negative nuclei ($n=60-89$ neurons; $p=3.7 \times 10^{-13}$, Student's unpaired t test). **B.** Left: phospho-dynamin 1 (PKC phosphorylation site) immunoreactivity in autaptic neurons from astrocyte-rich and astrocyte-poor cultures. Scale bar represents 20 μm . Right: quantification of background-subtracted phospho-dynamin 1 intensity from a single primary and two secondary dendrites averaged per neuron ($n=30$ neurons; $*p < 0.05$, Bonferroni corrected). As a positive control, we treated for 30 min with phorbol ester (1 μM PDBu) to increase PKC-dependent phosphorylation.

Discussion

Here we have demonstrated that astrocytes serve as permissive partners in the development of a form of persistent synaptic depression, presynaptic muting. In glutamatergic neurons from astrocyte-poor cultures, basal presynaptic function remained intact, suggesting that astrocytes modulate presynaptic plasticity competence differently than they regulate other types of presynaptic development. Astrocytic signaling was permissive, but not acutely instructive, for muting induction. Astrocyte-derived TSPs promoted the development of muting ability, as induced with varied stimuli, likely through $\alpha 2\delta$ -1 calcium channel subunits. Although the signaling pathways immediately downstream of $\alpha 2\delta$ -1 remain to be clarified, PKA appears to be an important downstream effector. PKA-dependent phosphorylation and synaptic behavior were abnormal in astrocyte-poor cultures but restored by TSP1 treatment, providing a potential mechanism by which TSP modulates muting competence.

TSPs are a class of proteins originally discovered in intact human blood platelets (Baenziger et al., 1971). In the brain, at least 4 TSPs are expressed in various regions and cell types (Iruela-Arispe et al., 1993; Adams and Tucker, 2000). TSPs are released from astrocytes and known to promote synaptogenesis (Asch et al., 1986; Christopherson et al., 2005; Xu et al., 2010), but because TSP-induced glutamate synapses in retinal ganglion cells are postsynaptically silent (Christopherson et al., 2005; Eroglu et al., 2009), TSPs may be involved mainly in presynaptic rather than postsynaptic differentiation. This is especially interesting given that TSPs act postsynaptically to produce these presynaptic effects (Eroglu et al., 2009; Xu et al., 2010). TSP increases the total number of synapses in retinal ganglion cells (Christopherson et al., 2005; Eroglu et al., 2009) but accelerates

synaptogenesis without altering total synapse number in hippocampal neurons (Xu et al., 2010). This regional difference in TSP's synaptogenic effects could explain why astrocyte deprivation failed to change total synapse number in our study of hippocampal neurons and why these effects of TSP on hippocampal plasticity have been previously overlooked. Because TSP expression increases after injury and is important for behavioral recovery after stroke (Lin et al., 2003; Liauw et al., 2008), TSP may be important generally for adaptive neuronal responses. Presynaptic muting, which reduced network activity during an excitatory insult during this study, and protected neurons against hypoxia in a prior study (Hogins et al., 2011), could be one such TSP-mediated synaptic ability contributing to adaptive neural responses during brain insults.

TSP promoted muting competence through its interaction with the $\alpha 2\delta$ -1 calcium channel subunit. This subunit serves as the TSP receptor for synaptogenesis in retinal ganglion cells (Eroglu et al., 2009) and as the receptor for the antinociceptive and anticonvulsant drug gabapentin (Gee et al., 1996). Gabapentin's therapeutic effects may arise from alterations in calcium channel organization (Arikkath and Campbell, 2003; Field et al., 2006; Bauer et al., 2009; Bauer et al., 2010; Hoppa et al., 2012), among other neuronal changes (Freiman et al., 2001; Gu and Huang, 2001; Stefani et al., 2001; Surges et al., 2003). Gabapentin binding to $\alpha 2\delta$ -1 antagonizes TSP-induced synaptic development in retinal ganglion neurons (Eroglu et al., 2009), and we found that gabapentin prevented the development of muting competence in hippocampal neurons, implicating TSP binding to $\alpha 2\delta$ -1 in this effect.

Muting incompetence due to gabapentin could also have clinical implications. Previous studies have demonstrated a wide variety of effects of gabapentin on synaptic

and behavioral plasticity including no effect (Cilio et al., 2001; Heidegger et al., 2010), reduced plasticity (Blake et al., 2007; Eroglu et al., 2009; Kurokawa et al., 2011), and enhanced learning (Buccafusco et al., 2010). In our study gabapentin blocked adaptive network changes in response to a stimulus known to selectively induce muting. Although we saw a non-significant increase in total network activity after gabapentin treatment, this likely does not explain the lack of muting since muting is promoted by increased network activity (Moulder et al., 2004; Moulder et al., 2006). Our study and a previous study (Eroglu et al., 2009) suggest that synaptogenic periods might be particularly vulnerable to unintended effects of gabapentin.

Our work implicates PKA signaling in TSP's promotion of muting competence. $G_{i/o}$ -linked receptor agonists and PKA inhibitors, which normally produce muting (Moulder et al., 2008; Crawford et al., 2011), persistently potentiated EPSCs in astrocyte-poor cultures. Additionally, PKA substrates in the presynaptic terminal and the nucleus were hyper-phosphorylated in astrocyte-deprived cultures, indicating an abnormal increase in PKA site phosphorylation or a decrease in dephosphorylation. This surprising synaptic facilitation may suggest that multiple cAMP-dependent pathways regulate synaptic transmission. It is possible that PKA inhibition normally suppresses transmission by dominating or masking other potentiating effects that are then revealed in the context of astrocyte deprivation and hyper-phosphorylated PKA substrates, although our work does not definitively address whether these changes alter synaptic transmission presynaptically, postsynaptically, or both. Astrocyte-released TSPs, therefore, may restrain PKA activity, or phosphorylation levels of downstream substrates, during synapse development so that PKA inhibition can produce a meaningful decrease in

synaptic function upon exposure to the depolarization challenge. Other models to explain these results are possible, and future work may distinguish them.

In addition to effects on synaptic plasticity, we also observed effects of astrocyte deprivation on basal postsynaptic function. Neuronal responses to AMPA and NMDA receptor agonists were depressed in parallel, supporting prior work suggesting that astrocytes are important for postsynaptic development (Beattie et al., 2002; Stellwagen and Malenka, 2006; Perea et al., 2009; Sullivan et al., 2011). Because neurons near astrocytes in astrocyte-poor cultures produced EPSCs with the same amplitude as those that were not, this postsynaptic effect of astrocytes likely resulted from a deficit in global astrocytic signaling rather than a deficit in local signaling. Astrocyte-derived TNF α controls postsynaptic receptor levels (Stellwagen et al., 2005; Stellwagen and Malenka, 2006; Steinmetz and Turrigiano, 2010), but TNF α did not alter muting competence in our study, suggesting that the reduction in postsynaptic receptors was not responsible for the muting deficit in astrocyte-deprived cultures. Additional evidence that postsynaptic receptors do not mediate muting includes current and prior work demonstrating muting in the presence of complete blockade of AMPA and NMDA receptor function (Moulder et al., 2004; Moulder et al., 2006) and the failure to mute in older, astrocyte-deprived cultures with larger basal EPSCs. Our results do not exclude a role for TSP and $\alpha 2\delta$ -1 in basal postsynaptic development, however, but TSP's role in muting appears independent of postsynaptic receptor function.

In summary, astrocyte-derived TSPs permit presynaptic muting at hippocampal glutamate terminals. TSPs likely act through $\alpha 2\delta$ -1 binding to normalize PKA signaling in developing synapses. These results reveal a novel mechanism by which glial-neuronal

communication controls adaptive synaptic malleability.

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Chapter 5

Discussion and future directions

Summary and significance

Synapses are critical points of communication between neurons and, therefore, mediate information transfer throughout the nervous system. Because of this vital role in neurobiological function, synaptic dysfunction likely contributes to many neurodevelopmental and neuropsychiatric disorders (Johnson et al., 2008; Betancur et al., 2009; Waites and Garner, 2011; Bhakar et al., 2012). Understanding synaptic function and modulation, therefore, is a fundamental goal in neuroscience that has the potential to inform processes mediating learning, behavior, and pathology. Presynaptic muting of glutamate release in response to strong depolarization or prolonged increases in neuronal activity is one such form of synaptic modulation that is poorly understood. This dissertation project aimed to clarify some of the mechanisms mediating muting induction in order to better understand adaptive synaptic phenomena.

Calcium is not necessary for muting induction:

In Chapter 2, I explored how introducing ligand-gated cation channels into neurons alters synaptic and neuronal function. I transfected cultured hippocampal neurons with TRPV1 or TRPM8 channels, which are thermosensitive cation channels important in sensory function (Talavera et al., 2008), with the original intention of controlling the activity of individual neurons in a cellular network. TRPV1-transfected neurons responded robustly to a saturating concentration of the TRPV1 agonist capsaicin. Similarly, TRPM8-transfected neurons responded robustly to menthol, a TRPM8 agonist. Non-transfected neurons did not respond to either capsaicin or menthol application. Prolonged ligand application reduced, but did not eliminate, channel-associated current,

suggesting that long-term application of agonists could be used effectively for tonic depolarization of a subset of neurons within a neuronal network. Although optogenetic strategies have become increasingly popular for manipulating activity of individual neurons within a neural network (Fenno et al., 2011; Tye and Deisseroth, 2012), this ligand-gated ion channel technique could have advantages in particular model systems. For example, neurons located far from each other could be activated with the same stimulus via widespread agonist application, and equipment and materials may be less costly than with optogenetics. Recently, exogenous expression of TRPV1 was used to control activity of neurons *in vivo* in mice (Arenkiel et al., 2008; Guler et al., 2012), demonstrating the promise of this technique for selectively controlling network activity in large, intact networks.

One important observation made after transfection with TRP channels was that TRPV1-transfected neurons expressed a basal leak current that was larger than that expressed in TRPM8-transfected and non-transfected neurons. This current was reduced with the TRP channel blocker ruthenium red, suggesting that it arises from the expression of TRPV1 and not from an artifact of the transfection. TRPV1, therefore, may be activated endogenously in our preparation, which is plausible given TRPV1's many known activating stimuli that could be present in our culture conditions (Schumacher, 2010; Vay et al., 2012). Additionally, I saw evidence of more agonist-induced toxicity in TRPV1-transfected than TRPM8-transfected neurons. After a 4 h exposure to agonist, a higher percentage of TRPV1-transfected neurons, but not TRPM8-transfected neurons, were unhealthy. This TRPV1 activation-associated toxicity was reduced in calcium-free extracellular medium, suggesting that calcium influx contributed to cell damage and

death. This dependence on calcium is consistent with prior literature exploring excitotoxicity mechanisms (Choi, 1987, 1992). TRPV1-transfected neurons may be more sensitive to calcium-dependent damage than TRPM8-transfected neurons due to TRPV1's higher relative calcium permeability as well as its tendency dilate after prolonged activation (Caterina et al., 1997; McKemy et al., 2002; Meyers et al., 2003; Chung et al., 2008). Calcium is not just an important component of excitotoxic signaling cascades; it is also an important induction signal for prolonged forms of synaptic plasticity like long-term depression and long-term potentiation (Malenka, 1991; Malenka, 1994; Teyler et al., 1994). A leak of calcium ions into the cell, therefore, may have explained the depressed autaptic, action potential-evoked postsynaptic currents (PSCs) in TRPV1-transfected neurons compared to TRPM8-transfected and synaptophysin-YFP-transfected neurons. This synaptic depression was not explained by transfection-induced alterations in synapse formation because both TRPV1- and TRPM8-transfected neurons released transmitter upon agonist exposure, as measured by miniature PSCs onto a postsynaptic neuron. I hypothesized, therefore, that influx of calcium through TRPV1 under basal conditions induced presynaptic muting. Because of the wide range of depolarizing stimuli that induce muting (Moulder et al., 2004; Moulder et al., 2006; Moulder et al., 2008; Hogins et al., 2011), it is plausible that depolarization from calcium influx contributes to muting induction. The role of calcium in muting induction and in the observed synaptic depression was not further clarified in this study, however, but future work should ask whether this basal EPSC depression in TRPV1-transfected neurons arose from muting. Future work should also clarify whether muting is cell autonomous by activating single neurons, potentially via menthol application to TRPM8-

transfected neurons, and assessing if muting is induced in the absence of input from other cells.

To elucidate whether muting requires calcium, I prevented calcium signaling during muting induction as described in Chapter 3. I used a 4 h high extracellular potassium stimulation to depolarize neurons and induce muting in the absence of other, confounding neuronal and synaptic changes. Buffering extracellular calcium with the slow calcium chelator EGTA did not prevent muting, as assessed by action potential-evoked excitatory PSCs (EPSCs) in autaptic neurons and by FM1-43 dye uptake into presynaptic terminals. This suggested that calcium entry from the extracellular space during neuronal depolarization is not required for muting induction. This did not, however, rule out the possibility that calcium from other sources, like intracellular calcium stores, contributed to muting induction. To prevent all intracellular calcium signaling, I loaded cells with the fast calcium chelator BAPTA by applying the extracellular precursor BAPTA-AM. BAPTA, although capable of preventing fast, calcium-dependent vesicle fusion in response to action potentials, did not prevent muting induction, as assessed by calcium-independent hypertonic sucrose-evoked EPSCs. As mentioned in Chapter 3, many forms of synaptic plasticity, especially those that are slowly and persistently induced, require calcium signaling (Wigstrom et al., 1979; Wickens and Abraham, 1991; Xie et al., 1992; Tong et al., 1996; Patenaude et al., 2003; Frank et al., 2006; Kellogg et al., 2009; Turrigiano, 2012). These surprising results suggested that muting induction is calcium-independent. Muting, therefore, is a unique form of synaptic plasticity that uses novel, calcium-independent induction mechanisms. Calcium-independent, long-term synaptic plasticity is rarely described, but a few known

forms are dependent on activation of metabotropic glutamate receptors (Fitzjohn et al., 2001; Ireland and Abraham, 2009; Kasten et al., 2012). I hypothesized, therefore that muting depends on calcium-independent release of a ligand that activates surface receptors important for modulating synaptic function.

G-protein signaling is required for muting induction:

Because muting did not require calcium, many potential molecular mediators of muting induction were excluded as candidates. For example, ligands released via calcium-dependent vesicle fusion, like many neurotransmitters and neuromodulators, are likely not involved in muting induction. Our lab recently showed, however, that levels of cyclic adenosine monophosphate (cAMP) correlate with the percentage of active glutamatergic synapses (Moulder et al., 2008), so cAMP modulators not dependent on calcium signaling remained viable candidates. It was unclear from this study, however, if reduced cAMP levels were instructive for depolarization-induced muting and, if so, which upstream signaling cascades mediate this cAMP change. In Chapter 3 I asked whether inhibitory G-proteins, which are activated through calcium-dependent and calcium-independent mechanisms to reduce cAMP levels and can mediate long-term synaptic plasticity (Hanoune and Defer, 2001; Poser and Storm, 2001; Brown and Sihra, 2008), mediate muting induction. Pertussis toxin, which prevents inhibitory G-protein signaling, blocked muting induction, as assessed with autaptic action potential-evoked EPSCs and FM1-43 dye uptake into glutamatergic terminals. This supported my hypothesis that inhibitory G-proteins are necessary for muting induction, but it did not clarify which G-proteins are activated and how.

To probe whether particular G-protein-coupled receptors (GPCRs) upstream of inhibitory G-proteins mediate muting induction, I asked if activation of inhibitory G-proteins by GPCR agonists was sufficient to induce muting. Adenosine and CCPA, which are nonselective and selective A1 adenosine receptor agonists, respectively, induced muting, as evidenced by persistent EPSC depression and a decrease in the percentage of glutamatergic terminals that labeled with FM1-43. Baclofen, a GABA_B receptor agonist, also produced muting. These results supported the hypothesis that G-protein activation produces muting. Interestingly, agonists for other GPCRs (CB1 cannabinoid and metabotropic glutamate) and kainate receptors, which can have metabotropic actions (Huettner, 2003), did not produce muting. This receptor behavior suggests some selectivity in the signaling cascades responsible for muting induction. Additionally, application of baclofen and CCPA together produced no more muting than either agonist alone, which would suggest that prolonged A1 and GABA_B receptor activation converge on the same downstream targets. Future work should clarify whether G-protein cascades activated by A1 and GABA_B receptors differ from those of metabotropic glutamate, CB1, and kainate receptors via molecular identity, activation levels, or spatial organization. For example, I showed in Chapter 3 that muting produced by A1 and GABA_B receptors was prevented by MG-132, a proteasome inhibitor. This result suggested that GPCR agonist-induced muting requires proteasome activity and aligned with prior published evidence from our lab showing that depolarization-induced muting requires proteasome activity (Jiang et al., 2010). Proteasome activation could be induced more strongly by A1 and GABA_B receptors than by metabotropic glutamate, CB1, and kainate receptors, but more experiments measuring proteasome activity during

receptor activation would be required to substantiate this hypothesis. One complication is that the magnitude of muting was smaller after A1 and GABA_B activation than after depolarization challenges. It is possible that proteasome activity is more weakly activated by agonists for these GPCRs than by depolarization or that depolarization activates additional signaling cascades beyond A1 and GABA_B-linked G-proteins. Pertussis toxin's ability to prevent muting suggested that G-protein-dependent cascades are responsible for muting, but these experiments did not clarify whether A1 and GABA_B receptors are the receptors activated by depolarization.

To ask which GPCR is responsible for muting induction, my initial strategy was to test likely candidates individually. Blocking metabotropic glutamate, CB1 cannabinoid, A1 adenosine, GABA_B, and kainate receptors individually with pharmacological agents, however, did not prevent muting after a depolarization challenge. This was surprising given A1 and GABA_B receptor agonists' ability to induce muting and suggested either that these receptors are not responsible for muting induction or that these receptors each contribute only a small fraction of the muting observed after the depolarization challenge. To test whether synergistic activation of multiple GPCRs contributes to muting induction, I pharmacologically blocked multiple receptors simultaneously. Blocking multiple receptors did not prevent muting induction, suggesting that none of the tested receptors are required for muting induction. As discussed in Chapter 3, this leaves some interesting questions unanswered, including whether other, untested receptors are required for muting induction or whether receptor-independent signals, like activators of G-protein signaling (AGS proteins), are responsible. One possibility is that an orphan GPCR or known inhibitory GPCRs like

neuropeptide Y or somatostatin receptors are responsible (Herzog et al., 1992; Cervia et al., 2003), but alternatively GPCR activation could be mediated or facilitated by voltage instead of a ligand (Ben-Chaim et al., 2006; Parnas and Parnas, 2007; Kupchik et al., 2011). Another possibility is that receptor-independent activation is responsible. If AGS proteins were tested for their role in muting, then AGS1 would be a good candidate because it is sensitive to pertussis toxin and activates inhibitory G-protein signaling cascades (Cismowski et al., 1999; Cismowski et al., 2000; Graham et al., 2004; Blumer et al., 2007). Clarifying the G-protein activation mechanism employed during muting induction remains an important goal.

Astrocytic signaling is required for the development of muting competence:

Because it was not known which receptor, if any, mediates muting induction, no additional clues were available to answer whether muting is cell autonomous. If muting requires GPCR activation, then this implies that a ligand is released extracellularly, likely from a different cell than the one undergoing muting, and binds to the GPCR; however, receptor-independent or ligand-independent activation could occur through cell autonomous signaling cascades. At this point, evidence for a cell autonomous mechanism included the expression of muting in autaptic neurons since autaptic neurons are physically isolated from other neurons. Additionally, intercellular communication during muting induction through calcium-dependent release of a GPCR ligand is unlikely because muting is calcium-independent and not induced via likely candidate GPCRs. Depolarization with high extracellular potassium, which depolarizes neurons above a membrane potential at which action potentials are blocked, and action potential stimulation, which repeatedly but briefly depolarizes neurons, both induce muting

(Moulder et al., 2004; Moulder et al., 2006), so voltage could serve as a cell autonomous induction signal. Arguments against cell autonomy, however, included the potential for retrograde messengers to pass from postsynaptic membranes to presynaptic terminals in autaptic neurons as well as the possibility that astrocytes acutely release gliotransmitters that modulate autaptic neuron behavior. Clarifying whether muting is cell autonomous could pinpoint the source of muting induction and narrow the list of candidate activators of G-protein signaling during depolarization. I, therefore, tested whether astrocytes are required for muting induction.

If muting occurs without communication with astrocytes, then muting induction may be either a cell autonomous phenomenon or one that requires local retrograde signaling at the synapse; in contrast, if muting is absent without astrocytes, then intercellular communication is required for muting induction. I tested for astrocytic contributions to muting induction by eliminating astrocytes from astrocyte-neuron co-cultures. As described in Chapter 4, I killed astrocytes using light aldehyde or ethanol fixation prior to neuronal plating. This technique allowed for the reduction of live astrocytes in the cultures while otherwise maintaining the same culture conditions as those used in the previous studies described here. Other methods for astrocyte elimination like glial toxins (Takada and Hattori, 1986; Hassel et al., 1992; Swanson and Graham, 1994; Menard et al., 1997), adjusting culture substrates and media to discourage astrocyte adhesion (Brewer et al., 1993; Ghosh et al., 1997; Recknor et al., 2004; Peretz et al., 2007), or genetic models preventing gliotransmission (Zhang et al., 2004; Li et al., 2005; Petravic et al., 2008) have clear disadvantages including loss of astrocyte tissue adhered to the culture dish, which serves as the substrate for neuronal attachment, or

prevention of only some subtypes of astrocyte-neuron communication. In Chapter 4, I showed that astrocyte fixation efficiently killed astrocytes in our cultures without abolishing autaptic neuron adhesion and development, validating this method as a way to probe the effects of astrocyte deficiency on neuronal and synaptic function.

Elimination of astrocytes from the co-culture network prior to synapse maturation prevented muting induction in response to depolarization, GPCR agonists, and cAMP signaling antagonists, suggesting that astrocytes are important for this form of synaptic plasticity. This failure to silence in astrocyte-poor cultures was not linked to an obvious deficit in presynaptic function; the probability of vesicle release, number of presynaptic terminals per neuron, number of vesicles per synapse, neuronal input resistance, and percentage of active glutamatergic synapses were not detectably different in astrocyte-poor cultures. Allowing the astrocyte-poor cultures to mature longer also did not restore muting, suggesting that the deficiency is fundamental to the lack of astrocytes instead of due to delayed development. A postsynaptic change occurred in astrocyte-poor cultures, however, as evidenced by the decreased EPSC amplitude that was linked to diminished AMPA and NMDA receptor function, which is consistent with prior literature (Beattie et al., 2002; Stellwagen and Malenka, 2006; Perea et al., 2009; Sullivan et al., 2011). The similar EPSC amplitudes between neurons from live (calcein-positive) astrocyte islands and dead (calcein-negative) astrocyte islands in astrocyte-poor cultures argued that the basal EPSC amplitude decrease was due to loss of global astrocytic cues rather than loss of local astrocytic cues. It was unclear if this postsynaptic deficiency indirectly altered presynaptic plasticity; for example, altered retrograde signaling from molecules released by the postsynaptic terminal that modulate presynaptic function may cause presynaptic

changes. I demonstrated, however, that young neurons from astrocyte-rich cultures with smaller basal EPSC amplitudes expressed muting while older neurons from astrocyte-poor cultures with larger basal EPSC amplitudes did not express muting. These results suggested that the basal EPSC amplitude does not determine muting competence, although this did not distinguish the contribution of total glutamate receptor levels to basal EPSC amplitude from that of total synapse number. Because of this evidence, a postsynaptic explanation for the loss of presynaptic plasticity in astrocyte-poor cultures seemed unlikely. Reduced astrocytic signaling, therefore, seems to unmask a deficit in presynaptic plasticity without altering presynaptic function *per se*, which may be why this synaptic effect has been overlooked in prior literature.

To clarify whether acute, instructive or global, permissive astrocytic signals were responsible for muting, I applied astrocyte-conditioned medium at different times relative to the depolarization challenge. When present for multiple days prior to muting induction attempts, astrocyte-conditioned medium rescued muting. Muting was also rescued by days of astrocyte-conditioned medium exposure even when replaced with non-conditioned medium during the depolarization challenge. The presence of muting in the absence of acute astrocytic signaling suggested that soluble factors released by astrocytes early in synaptic development permit muting competence. Astrocytes were not instructive for muting, arguing against the hypothesis that gliotransmission is involved in muting induction. This was supported by another experiment in which astrocyte-conditioned medium added only during the depolarization challenge did not restore muting induction. It appeared, therefore, that astrocytic factors promote muting competence via synaptic development, and synapses with a history of astrocyte signaling

do not require further astrocytic signaling to induce muting. This finding was especially interesting and suggested that astrocytes are metaplastic modulators of synapses; factors released by astrocytes mediate the synapse's ability to undergo future plasticity.

Astrocytes have a complicated relationship with muting; therefore, muting is not clearly cell autonomous or cell non-autonomous. Intercellular communication was clearly required for muting competence to develop in the presynaptic terminal, but synapses with a history of astrocytic signaling did not require exposure to acute astrocytic factors for plasticity induction. Cell autonomous mechanisms may be activated upon exposure to the depolarization challenge once non-cell autonomous signals have successfully promoted maturation, but dilute global signals released by the sparse neurons in the culture dish or retrograde signaling from postsynaptic compartments have not been ruled out entirely. Together, these results highlighted an important and novel role for astrocytes in synaptic development and narrowed the possible cellular sources of muting to either cell autonomous or a small range of cell non-autonomous mechanisms.

This novel role of astrocytes in the development of muting competence prompted a search for the molecular signals responsible. As described in Chapter 4, one major candidate was thrombospondin, a matricellular glycoprotein released by astrocytes that was recently found to be important for synapse development (Asch et al., 1986; Christopherson et al., 2005; Eroglu et al., 2009; Xu et al., 2010). Thrombospondins promote glutamatergic synaptogenesis in retinal ganglion neurons via binding to the structural calcium channel subunit $\alpha 2\delta$ -1 on the postsynaptic membrane (Christopherson et al., 2005; Eroglu et al., 2009). Interestingly, these thrombospondin-induced synapses are presynaptically functional but postsynaptically silent, meaning that AMPA receptors

are absent from the postsynaptic membrane (Christopherson et al., 2005). This suggests that thrombospondin specifically promotes presynaptic development. In hippocampal neurons, however, thrombospondins appear to modulate the speed of synaptogenesis, but not the total number of synapses that develop, via binding to the postsynaptic cell adhesion protein neuroligin 1 (Xu et al., 2010). These findings from hippocampal neurons suggesting that thrombospondin is not required for synaptogenesis may explain why astrocyte deficiency did not reduce the total number of presynaptic terminals during experiments performed in Chapter 4. Thrombospondin, therefore, was an excellent candidate for modulating presynaptic plasticity. Thrombospondin application to astrocyte-poor cultures a few days prior to the depolarization challenge rescued muting induction. Another astrocytic factor important for synaptic plasticity, tumor necrosis factor α , did not promote muting competence, suggesting that boosting synaptic function does not promote muting competence non-selectively. Although these results did not rule out a contribution to muting competence from other astrocyte-derived factors, they did suggest that thrombospondins are a class of molecules capable of mediating the development of muting ability.

To clarify whether thrombospondin endogenously mediates muting competence, I blocked thrombospondin signaling during synapse development. Prior literature suggests that thrombospondin's synaptogenic effects occur through activation of $\alpha 2\delta$ -1 calcium channel subunits or through neuroligin 1 (Eroglu et al., 2009; Xu et al., 2010), but it was unclear whether either of these receptors was recruited for thrombospondin's effects on synaptic plasticity competence. One pharmacological tool that allowed us to test the role of $\alpha 2\delta$ -1 in muting was the clinically used drug gabapentin. Gabapentin is known to bind

to $\alpha 2\delta$ -1 and prevent thrombospondin's synaptogenic effects in retinal ganglion cells (Gee et al., 1996; Eroglu et al., 2009). Adding gabapentin to astrocyte-conditioned medium prevented muting competence from developing in astrocyte-poor cultures. Gabapentin also prevented muting competence in astrocyte-rich cultures if the cultures were treated with fresh drug daily. These effects of gabapentin not only implicated $\alpha 2\delta$ -1 as the thrombospondin receptor that promotes muting competence, but they also implied that thrombospondin is a major, if not the only, contributor to muting competence in our cultures. Future work should probe the role of neuroligin 1 in muting competence, however, since it was not tested in these studies.

One potentially translational finding from Chapter 4 was that gabapentin altered adaptive changes in network activity. In mass cultures, where large networks of neurons develop, the depolarization challenge silenced excitatory and inhibitory spontaneous network activity. This was consistent with muting induction and subsequent failure of excitatory signaling, which likely caused a parallel reduction in spontaneous inhibitory signaling. This decrease in spontaneous activity after an excitatory stimulus further suggested that muting is an adaptive change; as shown in the Appendix (Hogins et al., 2011), muting is neuroprotective against excitotoxic insults like hypoxia, likely through dampened neuronal excitation. After chronic gabapentin treatment, however, this adaptive network change was absent. Interestingly, a non-significant increase in total network activity occurred after gabapentin treatment, potentially due to its effects on ionic conductances (Freiman et al., 2001; Stefani et al., 2001; Surges et al., 2003; Hoppa et al., 2012). It is unclear whether these ionic conductances modulate muting competence, but because thrombospondin promoted muting competence during the study,

it is likely that gabapentin's property as a thrombospondin receptor antagonist played a large role. The prevention of muting and adaptive network changes by gabapentin could have broader implications for patients treated with this drug. For example, children still undergoing intense synapse development could be more severely impaired than adults by gabapentin treatment because thrombospondin signaling appears important for synapse maturation. Also, patients taking gabapentin could experience additional neuronal toxicity during excitatory insults like stroke due to the lack of homeostatic network dampening during the insult. Future work should clarify whether the lack of adaptive plasticity in gabapentin-treated neurons renders networks more vulnerable to excitotoxic death, but the results described here indicate that gabapentin treatment should be employed judiciously.

The synapse-modifying signaling cascades downstream of thrombospondin-bound $\alpha\delta$ -1 were unknown prior to the study described in Chapter 4. Application of GPCR agonists or a PKA inhibitor not only failed to induce muting but also increased autaptic EPSCs in astrocyte-poor cultures, suggesting that an abnormality developed along this signaling cascade due to the lack of astrocytes. Acute GPCR agonist effects, thought to work through $G\beta\gamma$ subunit signaling (Brown and Sihra, 2008), were intact in astrocyte-poor cultures, however. This result suggested that signaling downstream of $G\alpha$ was disrupted in astrocyte-poor cultures but that G-protein signaling itself was functional. PKA targets were over-phosphorylated in astrocyte-poor cultures while a PKC target displayed normal levels of phosphorylation, so PKA activation downstream of $G\alpha$ may be rampant, or dephosphorylation impaired, in the absence of astrocyte-derived thrombospondin. Tellingly, thrombospondin normalized PKA phosphorylation levels,

which means that one mechanism by which thrombospondin signaling promotes muting competence is likely by maintaining PKA phosphorylation within a functional dynamic range. In a few prior studies, PKA inhibitors have blocked gabapentin-mediated neuronal and behavioral phenotypes (Martin et al., 2002; Lee et al., 2008; Takasu et al., 2008; Takasu et al., 2009), suggesting that these phenotypes require PKA-dependent pathways. Facilitated signaling through $\alpha 2\delta$ -1, therefore, could conceivably inhibit PKA-dependent pathways. I hypothesize that the increased PKA substrate phosphorylation in astrocyte-poor cultures overly stabilizes presynaptic priming proteins and, thereby, prevents protein degradation and muting induction. Future work should clarify the signaling cascade responsible for PKA-related alterations downstream of $\alpha 2\delta$ -1; one plausible hypothesis is that enhanced calcium signaling through $\alpha 2\delta$ -1-dependent calcium channel assembly inhibits calcium-inhibited adenylyl cyclases (Hanoune and Defer, 2001; Hoppa et al., 2012), thereby reducing cAMP and PKA signaling. Overall, these experiments revealed a novel role for astrocytes in promoting synaptic plasticity competence, but not induction of plasticity *per se*, through release of thrombospondins that alter signaling cascades important for muting induction.

Current muting induction model:

In summary, muting is induced by a complicated interplay between the state of the synapse before an excitatory insult and the signaling cascades recruited by the insult. The current model of muting induction is displayed in Figure 1. This model states that this adaptive, reversible form of synaptic plasticity requires astrocytic release of thrombospondin during synapse development to activate $\alpha 2\delta$ -1 calcium channel subunits

and normalize PKA signaling. I hypothesize that PKA activity after thrombospondin signaling and $\alpha 2\delta$ -1 activation, therefore, is reduced and maintained within a dynamic range able to produce meaningful changes in protein phosphorylation. A strong depolarization challenge or prolonged increased activity that decreases cAMP signaling via inhibitory G-protein activation, therefore, is capable of reducing PKA activity enough to decrease vesicle priming protein phosphorylation levels and render these proteins vulnerable to proteasomal degradation. The parallel increase in proteasome activity from the excitatory stimulus then causes protein degradation and loss of vesicle priming, thereby muting the synapse.

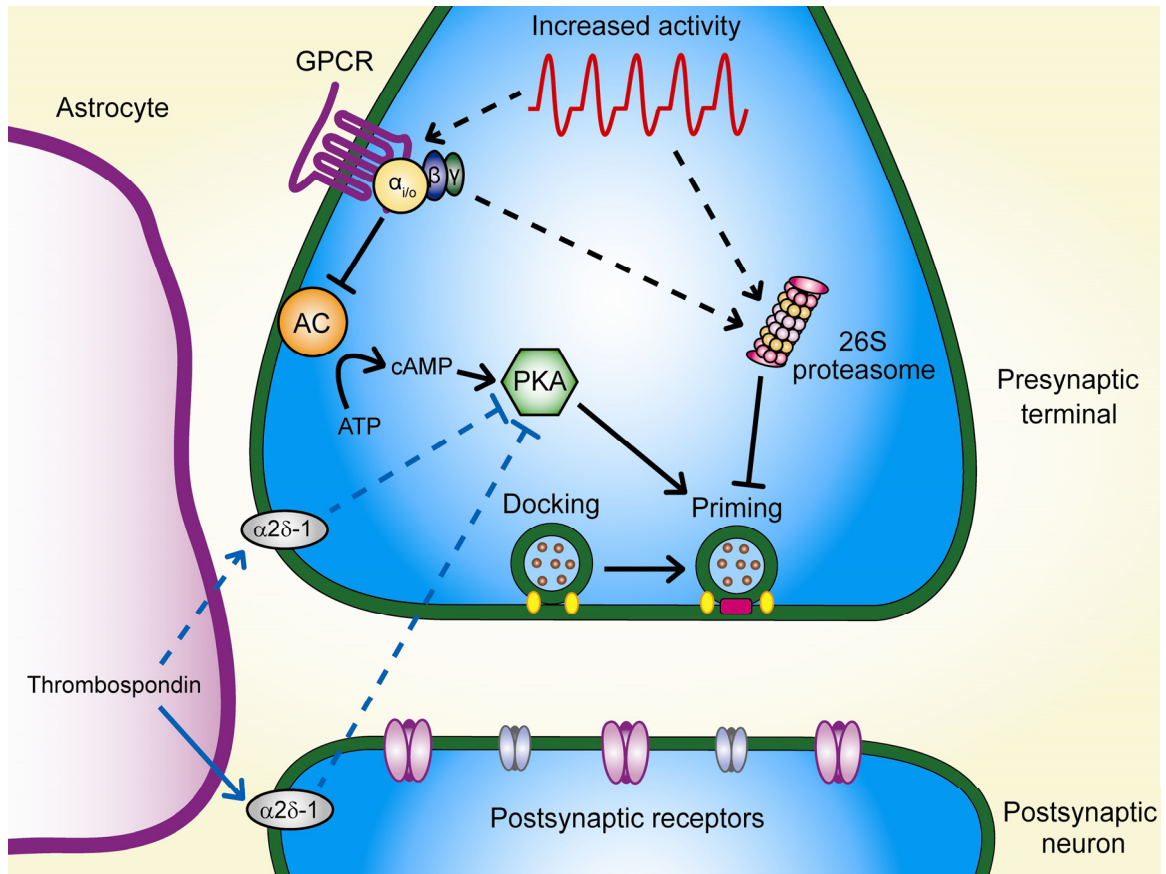


Figure 1. Signaling cascades participating in muting. During development (blue lines), astrocytic release of thrombospondin activates $\alpha 2\delta$ -1 receptors postsynaptically, presynaptically, or both, resulting in a normalization of PKA signaling. After synaptic maturity (black lines), prolonged strong depolarization or increased action potential firing induces presynaptic muting through activation of the ubiquitin-proteasome system and through activation of inhibitory G-proteins. Depolarization increases proteasome activity through unknown mechanisms, but both depolarization- and G-protein-coupled receptor (GPCR) agonist-induced muting require proteasome activity. Inhibitory actions of the $G\alpha$ subunit on adenylyl cyclase (AC) reduce cAMP and protein kinase A (PKA) signaling during muting induction. PKA phosphorylates presynaptic priming proteins like Rim1 α , a modification that may render Rim1 α resistant to proteasomal degradation; therefore, less Rim1 α phosphorylation is expected after depolarization. Increased proteasome activity, combined with a vulnerable presynaptic protein population, leads to priming protein degradation. This model provides a plausible mechanism for priming protein level reduction and muting induction by depolarization. Postsynaptic protein levels are unaltered by induction of presynaptic muting. Modified from Crawford and Mennerick, 2012.

Future directions

Interesting questions about the mechanisms, roles, and clinical potential of adaptive presynaptic muting emerged during these studies. Some of the immediate questions that should be answered include clarifying additional molecular mediators of muting competence or induction. For example, it remains unclear how thrombospondin signaling alters PKA activity. As described in the Summary and Significance, $\alpha 2\delta$ -1-dependent calcium channel assembly is one candidate downstream effect (Hoppa et al., 2012) since the role of calcium in the development of muting competence was never tested. Whether presynaptic or postsynaptic $\alpha 2\delta$ -1 is important for muting is also unclear. One potential strategy to test this would be to genetically knock down $\alpha 2\delta$ -1 in single neurons before assessing synaptic function on dendrites or axons of these neurons. This would reveal whether thrombospondin's effects are directly altering presynaptic function or indirectly altering presynaptic function through a retrograde signal. To clarify how quickly thrombospondin alters muting ability in presynaptic terminals, more experiments over differing time scales could be performed. For example, thrombospondin could be added 0-24 h prior to the depolarization challenge to ask whether shorter treatments promote muting competence. Additionally, thrombospondin's actions may have a critical window during synapse development. Would adding gabapentin later in development or for shorter periods of time prevent and/or reverse the development of muting competence? Do neurons from neonatal animals behave differently than those from adult animals? These studies could clarify the developmental role of thrombospondin and have important implications for gabapentin's clinical use.

It also remains unknown how inhibitory G-protein signaling is activated by

depolarization. If GPCRs are activated by a ligand during increased neuronal excitation, this ligand must be released by neurons, not astrocytes, since astrocytic signaling is not acutely required for muting induction. It is possible, however, that a non-canonical GPCR or G-protein activator is responsible. Genetic manipulations to knock down AGS proteins or other known GPCRs linked to inhibitory G-proteins in the hippocampus could identify the responsible G-protein activation mechanism, although this may require a high-throughput screening approach. Also, the dependence of muting on inhibitory G-protein signaling implies that stimulatory G-protein signaling (Gs) could mediate recovery from muting. Prior work has implicated calcium-dependent adenylyl cyclases in muting recovery (Moulder et al., 2008), but whether G-proteins, calcium, and/or another molecule are responsible for the adenylyl cyclase activation remains to be tested. The dependence of recovery from muting on PKA activity (Moulder et al., 2008) and the proteasome-dependence of muting (Jiang et al., 2010) also suggest that protein synthesis may be required, potentially through PKA-dependent activation of transcription factors like cAMP response element-binding protein (CREB; Sassone-Corsi, 1998). Unpublished data from our lab suggest that muting induction does not require protein synthesis, but recovery back to basal function does, and that phosphorylation of CREB by PKA is a plausible candidate mediator for this protein synthesis. Future work will have to explore the potentially causative role for CREB in recovery from muting.

As mentioned in the Summary and Significance, whether muting is cell autonomous remains unclear despite much work eliminating many intercellular signaling pathways during induction. Astrocytic signaling is not required during muting induction, as long as astrocytes are able to communicate with synapses prior to the induction

challenge, and autaptic neurons undergo muting. It is likely, therefore, that muting is cell autonomous, but the possibility of retrograde signaling from the postsynaptic neuron to the presynaptic neuron has not been excluded. To test for a retrograde signal, future work could genetically manipulate single neurons within a neural network. For example, optimizing transfection of TRPM8 in cultured hippocampal neurons would allow for studies that use tonic channel activation to depolarize neurons and attempt to induce muting. Other methods, like expression of channelrhodopsin and using light exposure to activate the channel (Fenno et al., 2011; Tye and Deisseroth, 2012), remain as alternatives to TRP channel transfection. If muting is induced selectively in one neuron on a two-neuron microisland using one of these techniques, for example, then paired recordings of these two neurons could parse out whether synapses onto the non-depolarized neuron or synapses onto the depolarized neuron undergo muting. Clarifying whether muting is cell autonomous or whether it requires a retrograde signal would narrow the list of candidate activators of G-protein signaling even further. Additionally, identifying the source of the signal to mute could have implications for how muting interacts with network activity. For example, does the neuron being overly activated prevent itself from further propagating the overexcitation, or does it signal to the neuron prior to it in the circuit to reduce its own overactivation? Although evidence currently supports the hypothesis that muting is cell autonomous (i.e. the presynaptic terminals within the neuron being activated are muted without intercellular communication), further work is required to clarify this definitively.

More fundamentally, the role of mute terminals in information processing in the nervous system remains unclear. For example, why is muting binary, in that the synapse

is entirely non-functional but structurally preserved? Because of this binary nature, does muting alter the electrical transfer of information in the same way as synaptic pruning but with the added benefit of saving time and cellular resources? Understanding the mechanisms involved in muting could lead to the ability to strategically silence particular synapses (within a single neuron, a single circuit, or an entire brain region) and observe the consequences on neuronal excitation, subcellular electrical summation, and network activation. It also remains unclear if the synapses that mute in response to depolarization are randomly distributed to dampen general excitability or strategically placed to modulate firing of particular postsynaptic neurons. The probability of vesicle release and readily releasable pool size increase with distance along the axon from the cell body in hippocampal neurons (Peng et al., 2012), so it is possible that the basal state of the synapse or its location could determine the probability that a synapse will undergo muting. One could measure muting and unmuteing using live-cell imaging to determine if the same terminals are vulnerable to muting with repeated depolarization challenges and whether the location along the axon or dendrite correlates with this tendency. Also, because cAMP is an important mediator of muting induction, cAMP levels could be measured in axonal compartments, using techniques like the FRET construct Epac1-camps (Nikolaev et al., 2004), to determine whether basal cAMP levels or the dynamic range of cAMP levels near particular synapses correlate with a tendency to mute. Alternatively, the total percentage of mute terminals, if distributed randomly, may correlate with somatic cAMP levels.

The location of mute terminals may also have important implications for how muting interacts with other forms of synaptic plasticity like homeostatic postsynaptic

receptor scaling or Hebbian long-term potentiation. Synaptic scaling purportedly acts at all synapses in a neuron (Turrigiano, 2012), but long-term potentiation is specific to synapses experiencing strong, correlated presynaptic and postsynaptic activity, leaving nearby synapses unaffected (Bear and Malenka, 1994; Kelleher et al., 2004). Muting would be predicted to enhance the effects of reduced postsynaptic receptor levels on overall network activity but may cancel the effects of Hebbian plasticity at particular synapses. Computer modeling would facilitate the study of how these forms of plasticity interact and what effects simultaneous inductions have on neural computation and network activity. As techniques evolve for inducing multiple forms of synaptic plasticity simultaneously or sequentially in living tissue, the physiological consequences of their interactions should be tested.

One major open question remaining about muting induction is what role it plays *in vivo*. In mammals, evidence determining whether muting even occurs *in vivo* is needed. At this time, work outside of cultured neuron networks, like in acute hippocampal slices (Moulder et al., 2004; Crawford and Mennerick, 2012; Chapter 1), have merely shown correlates of muting due to the technical limitations involved. Future directions should include techniques like high resolution microscopy of presynaptic vesicle function or sophisticated electrophysiological measures of individual neurons to measure muting *in vivo* (Kalb et al., 2004; DeWeese, 2007; Ako et al., 2011; Herzog et al., 2011; Kodandaramaiah et al., 2012). Once the presence of and mechanisms for *in vivo* muting have been established, then the field is poised for exploring how muting alters learning, behavior, or disease. For example, establishing whether muting occurs in all brain regions and neurotransmitter systems will be important for developing

hypotheses about its role in brain function. Because muting occurs in hippocampal glutamatergic neurons and has the potential to interact with Hebbian plasticity, it could disrupt or enhance learning and memory. To test this, muting would need to be induced *in vivo* prior to or during learning and memory tasks. Muting may also be regulated by sleep and circadian rhythms, as has been proposed for Hebbian plasticity (Tononi and Cirelli, 2006). Inducing muting during different phases of the circadian cycle, therefore, may cause different behavioral outcomes. Similarly, neurons may be more or less susceptible to muting early in development than later in development, so the effects of muting at different developmental ages should also be assessed. Potentially the most translational open question would be whether muting occurs in pathophysiological contexts. Do changes in muting levels mediate alterations in the balance of excitation and inhibition observed during disease (Eichler and Meier, 2008; Baroncelli et al., 2011; Kapogiannis and Mattson, 2011; Ramamoorthi and Lin, 2011), and could manipulating muting *in vivo* restore balance to unbalanced networks? Could clinical induction of muting be used to prevent or reduce damage from excitatory insults like stroke or seizure? Although these questions will not be answered immediately, clarifying the role of muting in cognition, learning, or disease will provide insight about the role of synapses in brain function.

Conclusions

Synaptic function and plasticity are vital to information processing within the nervous system. Presynaptic muting of glutamatergic synapses represents an underappreciated form of synaptic plasticity present in hippocampal glutamatergic neurons. For this dissertation project, I have clarified some of the molecular mediators of muting development and induction. Calcium, an important mediator of synaptic plasticity, was surprisingly not necessary for muting induction while inhibitory G-protein signaling, best known for its role in short-term plasticity, was necessary. Astrocyte-derived thrombospondins promoted muting competence of presynaptic terminals, but astrocytes were otherwise not necessary for muting induction. This work revealed novel mechanisms by which synapses are regulated and revealed some potentially translational implications of muting and the pharmacological agents that modulate it. Although much remains to be learned about the mechanisms responsible for muting, and the contexts under which they are recruited, this work has laid a strong foundation for future work in the field of synaptic development and plasticity.

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Appendix

Presynaptic silencing is an endogenous neuroprotectant during excitotoxic insults

This appendix contains a previously published manuscript:

Hogins J, Crawford DC, Jiang X, Mennerick S (2011). Presynaptic silencing is an endogenous neuroprotectant during excitotoxic insults. *Neurobiol Dis* 43(2): 516.

Author contributions for the citation above:

J.H. designed and performed experiments, analyzed data, and wrote the paper. D.C.C. designed and performed experiments and analyzed data. X.J. performed experiments and analyzed data. S.M. designed and performed experiments, analyzed data, and wrote the paper.

Abstract

Glutamate release is a root cause of acute and delayed neuronal damage in response to hypoxic/ischemic insults. Nevertheless, therapeutics that target the postsynaptic compartment have been disappointing clinically. Here we explored whether presynaptic silencing (muting) of glutamatergic terminals is sufficient to reduce excitotoxic damage resulting from hypoxia and oxygen/glucose deprivation. Our evidence suggests that strong depolarization, previously shown to mute glutamate synapses, protects neurons by a presynaptic mechanism that is sensitive to inhibition of the proteasome. Postsynaptic Ca^{2+} rises in response to glutamate application and toxicity in response to exogenous glutamate treatment were unaffected by depolarization preconditioning. These features strongly suggest that reduced glutamate release explains preconditioning protection. We addressed whether hypoxic depolarization itself induces presynaptic silencing, thereby participating in the damage threshold for hypoxic insult. Indeed, we found that the hypoxic insult increased the percentage of mute glutamate synapses in a proteasome-dependent manner. Furthermore, proteasome inhibition exacerbated neuronal loss to mild hypoxia and prevented hypoxia-induced muting. In total our results suggest that presynaptic silencing is an endogenous neuroprotective mechanism that could be exploited to reduce damage from insults involving excess synaptic glutamate release.

Introduction

For decades we have known that glutamate excitotoxicity (Olney et al., 1971) results in damage to neurons following a wide variety of insults including hypoxia, ischemia, seizures (Gidday, 2006; Goldberg and Choi, 1993; and Rothman, 1984), and neuropsychiatric disorders (Olney, 2003; and Zorumski and Olney, 1993). Therapeutic approaches have centered on blocking glutamate receptors and other downstream, postsynaptic targets involved in the excitotoxic cascade. Unfortunately, these approaches have proven disappointing in human studies because of poor efficacy or unacceptable side effects (Gidday, 2006; and Moskowitz et al., 2010). Thus fresh approaches and new basic insights are needed. One alternative might be to study and exploit endogenous homeostatic synaptic mechanisms as strategies. By augmenting endogenous pathways of adaptive synaptic plasticity, such strategies might circumvent some of the problems that have arisen with previous interventions.

We have been studying a form of persistent presynaptic plasticity involving depression of glutamate vesicle availability in hippocampal neurons. This depression is characterized by presynaptic silencing, or muting, that outlives the inducing stimulus. Muting is induced at glutamate, but not γ -aminobutyric acid (GABA), synapses by strong depolarization (Moulder et al., 2004). It reverses over several hours (Moulder et al., 2008) and is characterized by involvement of inhibitory G-protein signaling (Crawford et al., 2011) and the ubiquitin/proteasome system (UPS) (Jiang et al., 2010).

Two issues relevant to excitotoxic pathophysiology emerge from our previous observations. First, because the source of glutamate during excitotoxic insults may not be purely synaptic (Rossi et al., 2000), it is unclear whether presynaptic interventions, such

as induction of presynaptic muting, are effective neuroprotectants. Second, because presynaptic silencing has been induced only under controlled experimental conditions, it is unclear whether depolarizing insults such as hypoxia can induce muting rapidly and strongly enough to provide endogenous neuroprotection. If so, muting may normally help set the threshold for damage during insults. Support for these two ideas would help establish the plausibility of augmentation of presynaptic muting as a neuroprotective intervention and would offer new basic insights into the roles of synaptic plasticity in nervous system function and dysfunction.

Our results demonstrate that presynaptic silencing induced by strong depolarization protects neurons in *in vitro* hypoxia and oxygen-glucose deprivation (OGD) models, consistent with the idea that synaptic glutamate is important for the damage. Although depolarizing preconditioning paradigms have been shown to be neuroprotective in other models through postsynaptic mechanisms (Grabb and Choi, 1999; Grabb et al., 2002; and Meller et al., 2008), we show that presynaptic mechanisms, most likely involving presynaptic muting, are most important in our paradigm. Further, our results suggest that a hypoxic insult induces muting to limit damage. Proteasome inhibition, among other likely effects, prevents hypoxia-induced silencing and exacerbates damage through a presynaptic mechanism, suggesting that muting helps set the threshold for hypoxic damage. These results yield insights and suggest new approaches that might be exploited for benefit in disorders involving dysfunction of glutamate synapses.

Materials and methods

Cell culture:

Hippocampal cultures were prepared as described previously (Mennerick et al., 1995). In brief, dissected postnatal (postnatal days 0–3) male and female rat hippocampi were incubated with papain, mechanically dissociated, and plated at 650 cells/mm² on collagen substrate. Plating media consisted of Eagle's medium (Invitrogen) supplemented with heat-inactivated horse serum (5%), fetal bovine serum (5%), 17 mM glucose, 400 µM glutamine, 50 U/mL penicillin, and 50 g/mL streptomycin. Cultures were maintained at 37 °C in a humidified incubator with 5% CO₂/95% air. Cytosine arabinoside at 6.7 µM was added at 3–4 days after plating to inhibit cell division. At 1 day a media exchange was performed with Neurobasal medium (Invitrogen) plus B27 supplement. Cells were used for experiments at 13–15 days *in vitro*. Neurons exhibited increased sensitivity to all forms of glutamate toxicity with maturation.

Preconditioning:

Fresh filtered Neurobasal (Invitrogen) medium without L-glutamine was used as a base for preconditioning media and media exchanges. The original conditioned media was removed and replaced with the preconditioning media. The cultures were returned to their original media just before hypoxic exposure. For depolarizing preconditioning, the preconditioning media contained Neurobasal plus 30 mM KCl. Control preconditioning media included 30 mM NaCl in place of KCl as an equimolar non-depolarizing control. In our typical protocol, this resulted in control and experimental preconditioning

solutions that were ~ 60 mOsm hyperosmotic. To ensure that hypertonicity did not interact with the effects of depolarization, we performed a subset of preconditioning experiments in media that were isotonic (142 mM total monovalent cation concentration, KCl substitution for NaCl, $n = 5$ experiments). These experiments produced similar preconditioning protection as our standard protocol ($27.7 \pm 9.66\%$ cell survival with hypoxia, $62.8 \pm 3.58\%$ survival with hypoxia plus KCl preconditioning, compare with [Fig. 1](#)). We did not routinely perform experiments in the isotonic media because of the high expense of custom media preparation. Unless otherwise noted, all control and depolarizing preconditioning solutions also included $0.5 \mu\text{M}$ 2,3-dihydroxy-6-nitro-7-sulfonyl-benzo[f]quinoxaline (NBQX), and $25 \mu\text{M}$ D-2-amino-5-phosphonovalerate (D-APV) to prevent induction of glutamate-receptor dependent forms of preconditioning protection or plasticity during preconditioning. In some experiments we omitted extracellular Ca^{2+} (0.1 mM EGTA added to chelate residual Ca^{2+}) or included carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG-132; Sigma, $3 \mu\text{M}$) in preconditioning solutions. Unless otherwise noted, the media was exchanged immediately before hypoxic exposure to remove glutamate receptor antagonists and the preconditioning stimulus.

Hypoxia exposure:

A commercially available chamber (Billups-Rothenberg Company) was used for hypoxia induction and maintenance. Cells were incubated in a humidified environment saturated with 95% nitrogen and 5% CO_2 at 37°C for the specified amount of time (2–2.5 h depending on the experiment). The gas exchange was performed according to the specifications of the chamber manufacturer (flow of 20 L per minute for 4 min to achieve

100% gas exchange). After the insult, we removed cells from the chamber and incubated them under standard conditions until the cell death assay (24 h). When used during the insult to demonstrate neuroprotection, the glutamate receptor antagonists were 1 μ M NBQX and 100 μ M D-APV, but antagonists were not routinely present during hypoxia or OGD insults. For OGD experiments the method of oxygen deprivation was the same, and just prior to oxygen deprivation culture media was switched to Neurobasal without L-glutamine and without glucose. Control cultures received the same media with glucose (25 mM). Procedures for sham insults indicated in figures included all media changes and incubation times relevant to the insult conditions. For experiments in which exogenous glutamate exposure was used in place of hypoxia, media was exchanged with fresh Neurobasal without L-glutamine and with the indicated concentration of glutamate. The cultures were then returned to their original media for 24 h until the cell death assay was performed.

Cell death assay:

To assess cell death, trypan blue dye (Sigma) was used. 24 h after the insult, culture media was removed and replaced with 1 mL of 0.4% trypan blue dissolved in phosphate buffered saline. Cells were incubated in dye at 37 °C for 5 min and washed with phosphate buffered saline. Cultures were then fixed with 4% paraformaldehyde and 0.2% glutaraldehyde at room temperature. Cells were visualized with a 20 \times objective using both phase-contrast and brightfield microscopy to confirm healthy neuronal profiles (phase-contrast) and verify trypan blue uptake (brightfield). In one experiment the designation of healthy neurons was verified 24 h after hypoxia treatment by calcein AM

(acetoxymethyl) uptake (2 μ M calcein-AM incubation for 30 min). Cells deemed healthy by morphology under phase-contrast optics were always calcein positive ($n = 5$ fields, 20–40 cells per field). The total numbers of dead and intact neurons were counted and expressed as a percentage of trypan blue positive dead cells to total cells. The average of ten microscope fields for each condition was treated as a single data point for purposes of statistics.

Calcium indicators and imaging:

Fluo4-AM (high affinity indicator; Invitrogen) and Fluo3-FF-AM (low affinity indicator; Teflabs) fluorescent calcium indicators were used. We incubated neurons in preconditioning solutions as noted above. After the 4 h preconditioning period cells were incubated for 30 min at 37 °C in normal media containing 2 μ M of the AM indicator. 5–7 fields per condition were imaged. Fluorescence images were obtained with a CoolSnap ES2 camera (Photometrics) every 600 ms, using a fluorescein filter set and metal halide light source. We selected 3 cells randomly from each field from a phase contrast image, without reference to fluorescence images, for a total of 15–21 cells per condition as indicated. Regions of interest were analyzed in the soma cytoplasm adjacent to the nucleus. Background fluorescence was subtracted from each image using a cell-free region of the field. Image acquisition and analysis were performed using Metamorph (MDS).

FM1-43fx labeling, immunochemistry, and imaging:

Details have been previously published (Crawford et al., 2011). Briefly, cells

were removed from preconditioning (Fig. 2) or the hypoxia chamber (Fig. 7), and after a saline wash (~ 30 s) FM1-43fx (10 μ M; Invitrogen) was loaded into active presynaptic terminals during a brief (2 min) 45 mM KCl depolarization. vGluT-1 primary antibody (1:2000) was applied after aldehyde fixation, permeabilization, and block. Alexa Fluor 647 goat anti-guinea pig secondary antibody (Invitrogen) was used at 1:500.

Fluorescence images were acquired using a Nikon C1 confocal microscope and analyzed using Metamorph software. Parameters for image acquisition and analysis were constant for each independent experiment.

To identify active glutamatergic synapses, an observer naïve to the experimental conditions identified 10 vGluT-1-positive puncta as regions of interest from 5 fields for each experimental condition. Next, FM1-43fx images were thresholded, after which vGluT-1 regions were loaded into the FM1-43fx image. Active synapses were defined as puncta that reached or exceeded 10 thresholded pixels in the FM1-43fx image (Crawford et al., 2011).

Data analysis:

Data analysis was performed in Excel (Microsoft) and Sigma Plot 10.0 (Systat Software). Student's unpaired *t*-test was used to test for significance. Where indicated, the Bonferroni correction for multiple comparisons was used. Except where otherwise indicated, values for *n* in the text and figure legends represent independent experiments on separate cultures.

Materials:

All reagents were obtained from Sigma Chemical Company unless otherwise indicated. Culture media were from Invitrogen, guinea pig vGluT-1 antibody was from Chemicon.

Results

Hypoxic damage is attenuated by depolarization preconditioning:

We explored the hypothesis that strong depolarization, a stimulus that induces presynaptic muting in hippocampal neurons, protects neurons from subsequent hypoxic damage. Unlike other forms of preconditioning, which exhibit a latent period during induction of hours or days (Gidday, 2006; and Kitagawa et al., 1991), presynaptic muting is induced by strong depolarization, and recovers within a few hours of removal of the inducing stimulus (Moulder et al., 2004). Therefore, we designed our paradigm to capture the likely protective effect of presynaptic muting immediately after induction, without invoking previously described mechanisms of preconditioning protection with longer latencies. We incubated synaptically mature cultured hippocampal neurons (13–15 days *in vitro*) with 4 h of high KCl (30 mM), a stimulus that silences 75–80% of glutamate presynaptic terminals, or 4 h of NaCl (30 mM) as an osmotic, non-depolarizing control (Moulder et al., 2004). Both control preconditioning and depolarizing preconditioning were always performed in the presence of D-APV and NBQX to prevent glutamate receptor activation. This ensured that other forms of synaptic plasticity dependent on glutamate receptors were not activated by preconditioning; presynaptic silencing is not glutamate receptor-dependent (Crawford et al., 2011; Moulder et al., 2006; and Moulder et al., 2004). After preconditioning, the neurons were then removed from the preconditioning stimulus, including receptor antagonists, by media exchange and immediately exposed to hypoxia for 2.5 h. In addition to terminating the preconditioning stimulus, this media exchange also eliminated glutamate receptor antagonists and any

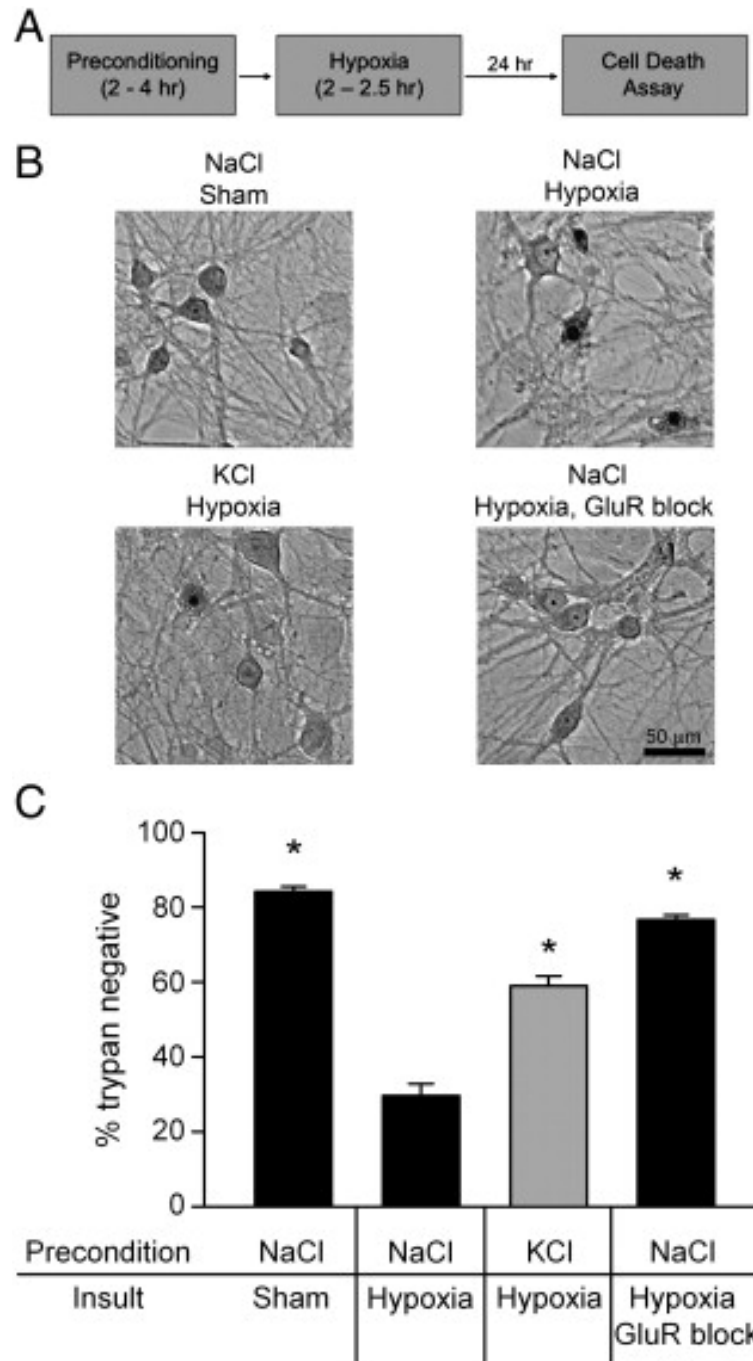


Figure 1. Depolarization preconditioning protects against hypoxia. **A.** Schematic of preconditioning/hypoxic exposure paradigm. **B.** Photomicrographs from a single experiment demonstrating depolarization protection. Upper left: brightfield image from a normoxic control stained with trypan blue 24 h post-preconditioning with 4 h of 30 mM NaCl (control preconditioning) and 2.5 h sham hypoxia. Upper right: a field from a dish preconditioned with 30 mM NaCl and subjected to 2.5 h hypoxia. Trypan blue positive pyknotic nuclei are apparent. Lower left: a field from a dish preconditioned with 30 mM KCl for 4 h and then subjected to 2.5 h hypoxia. Lower right: a protection control in which 1 μ M NBQX and 100 μ M D-APV, ionotropic glutamate receptor (GluR)

antagonists, were included in the hypoxia media. **C.** Summary of experiments like that depicted in panel B, showing protection afforded by KCl depolarization preconditioning. Cell survival is expressed as the percentage of trypan blue negative cells averaged over ten fields evaluated with a 20× objective ($n = 7$ independent experiments). Asterisks designate $p < 0.05$ compared with NaCl hypoxia (unpaired, two-tailed t -tests with Bonferroni correction for multiple comparisons). Gray bar emphasizes the major hypothesized result of KCl preconditioning protection.

contributions of substances secreted during preconditioning (see Materials and methods). Thus, only persisting cellular changes contributed to neuroprotection during hypoxia. We used trypan blue staining, a well-validated probe of membrane integrity, 24 h after the insult to assess cell death (Fig. 1A). With sham treatment, we found only mild attritional cell death ($84 \pm 1.3\%$ healthy neurons; Figs. 1B and C), consistent with previous work (Shute et al., 2005). In cells rendered hypoxic after control (NaCl) preconditioning, a 2.5 h hypoxic insult resulted in $30 \pm 3.2\%$ survival (Figs. 1B and C). By contrast, depolarizing preconditioning strongly and reliably protected neurons from hypoxia ($59 \pm 2.6\%$ survival, Figs. 1B and C). Postsynaptic glutamate receptor blockade during the insult nearly fully protected neurons ($77 \pm 1.4\%$ survival, Figs. 1B and C), as previously shown (Rothman, 1984), and verifying the pivotal role of glutamate release and glutamate receptor activation in hypoxic cell loss.

Depolarization preconditioning works through a presynaptic mechanism:

These results suggest that a stimulus known to induce presynaptic silencing protects neurons from damage by endogenous glutamate. To verify presynaptic muting by the preconditioning paradigm, we performed analysis of FM1-43fx labeling of glutamatergic presynaptic terminals (defined by vGluT-1 immunoreactivity). As previously observed (Crawford et al., 2011; Jiang et al., 2010; and Moulder et al., 2004), 4 h depolarization preconditioning silenced a majority of glutamate terminals (Figs. 2A and B). Because the FM1-43fx loading protocol used 2 min of strong depolarization to induce vesicle cycling, loading should overcome changes in vesicle release probability that could occur, for instance, by decreases in Ca^{2+} influx during dye loading. This

protocol has been shown to label the entire pool of vesicles capable of exo/endocytosis (Mozhayeva et al., 2002). Therefore, terminals appear functionally silenced in response to the preconditioning stimulus, a state that would be expected to effectively reduce glutamate release during major insults such as hypoxia. In this set of experiments we also noted a mild decrease in FM1-43fx uptake at remaining, active synapses (Fig. 2A), which could indicate a graded change in the number of releasable vesicles (Murthy et al., 2001).

We have previously shown that changes in FM1-43fx uptake quantitatively match depression of EPSCs, suggesting that postsynaptic changes do not contribute significantly to the effects of depolarization conditioning (Moulder et al., 2004). Furthermore, depolarization preconditioning does not induce detectable postsynaptic changes measured by mEPSC analysis, response to exogenous glutamate agonists, or AMPA receptor immunoreactivity (Moulder et al., 2006; and Moulder et al., 2004). These past experiments do not exclude the possibility that depolarization conditioning alters postsynaptic Ca^{2+} influx or handling downstream of receptor activation. To test whether depolarization preconditioning influences the postsynaptic Ca^{2+} signals presumably important for hypoxic damage (Choi, 1985; and Choi, 1987), we loaded cells with Fluo3-FF and then challenged depolarization-preconditioned cells and control cells with brief applications of 10 μM glutamate (Figs. 2C and D). Because cells were stimulated by exogenous glutamate applied to somatodendritic postsynaptic receptors, Ca^{2+} signals measured near the soma should reflect any changes in receptor-mediated Ca^{2+} influx or intracellular handling that might participate in neuroprotection. Preconditioned neurons did not differ in amplitude or half decay time ($t_{1/2}$) of somatic Ca^{2+} signals evoked by a 5 s application of 10 μM glutamate, indicating that preconditioning did not affect Ca^{2+}

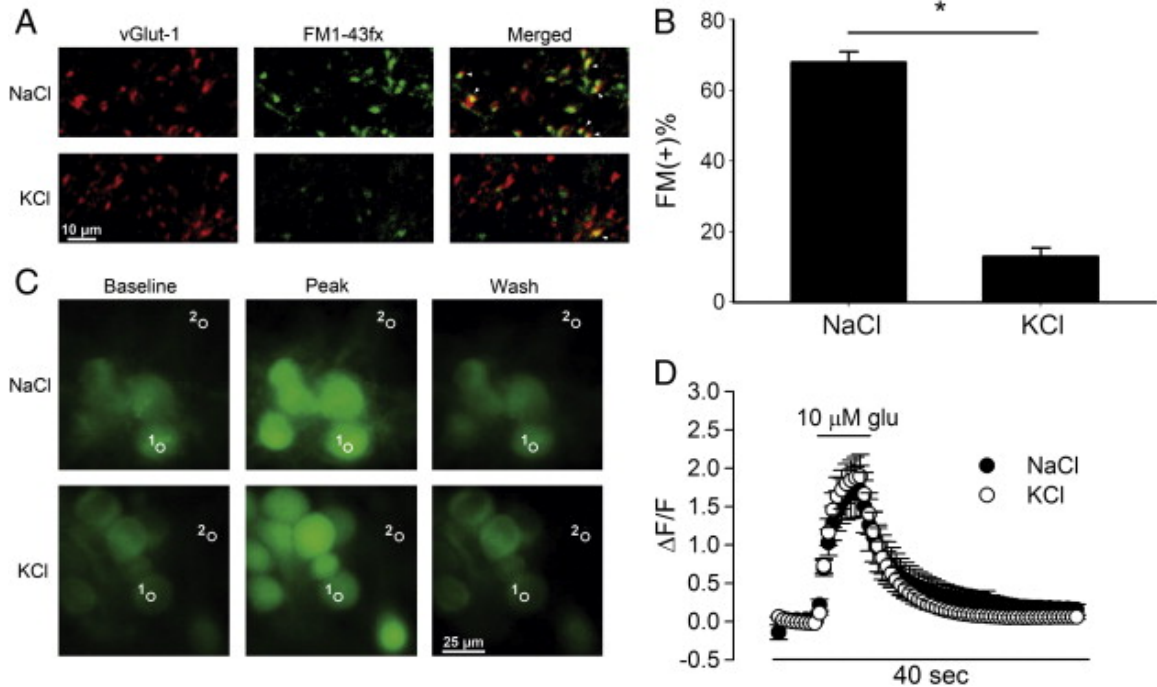


Figure 2. Preconditioning induces presynaptic muting but no detectable postsynaptic changes. **A.** Images of vGluT-1 positive puncta from representative fields from cultures preconditioned by 4 h KCl (30 mM) depolarization or by 30 mM NaCl control preconditioning. Middle panels show uptake of FM1-43fx during brief depolarization in the same field. The merged images reveal more inactive (FM1-43fx negative) vGluT-1 positive puncta after depolarizing preconditioning. Green = FM1-43fx. Red = vGluT-1. Red puncta with no green overlap are mute synapses, while yellow indicates overlap and active synapses. White arrowheads indicate examples of co-labeled, active glutamatergic synapses. **B.** The percentage of FM1-43fx positive (FM (+)) terminals is summarized from 5 experiments like that depicted in A. **C.** Representative fluorescence images from somatic Ca^{2+} signals measured in control ($n = 18$ cells in 6 fields) and depolarization-conditioned ($n = 15$ cells in 5 fields) cells. Cells were loaded with a 30 min bath application of 2 μM Fluo3-FF AM. Representative regions of interest (labeled with '1') show typical locations of measurement in the soma, chosen based on a phase contrast image (not shown). Dendrites and axons are below the plane of focus. Images of baseline (prior to glutamate perfusion), peak fluorescence (5 s following 10 μM glutamate onset), and return to baseline (30 s wash) fluorescence are shown. Region 2 indicates a region devoid of cells and typical of regions from which background fluorescence was measured. **D.** Summary of glutamate-evoked somatic intracellular Ca^{2+} signals measured over the entire experiment (67 images over 40 s) with control-conditioned and depolarization-conditioned cells overlaid.

handling or buffering in the postsynaptic compartment (peak $\Delta F/F = 1.8 \pm 0.4$, and $t_{1/2} = 3.3 \pm 0.6$ s for NaCl control; peak $\Delta F/F = 2.0 \pm 0.3$, and $t_{1/2} = 3.2 \pm 0.3$ s for KCl preconditioning, $p > 0.05$, $n = 18$ cells for control, $n = 15$ cells for KCl preconditioning; Figs. 2C and D). We used the low-affinity dye Fluo3-FF to prevent dye saturation, but to ensure that the indicator was not saturated by our glutamate challenge, we applied 100 μM glutamate to 15 additional cells and found that peak $\Delta F/F$ from these cells was 4.5 ± 0.6 , much larger than the 2.0 ± 0.3 $\Delta F/F$ average peak 10 μM glutamate signal, confirming the lack of dye saturation.

It is possible that the low-affinity indicator may not reveal differences in the decay phase of glutamate-induced Ca^{2+} rises, where the Ca^{2+} concentration falls below detection limits of the dye. Therefore, we examined decay $t_{1/2}$ values using the high-affinity indicator Fluo4-AM. As expected, the decays using the high-affinity dye were slower than those observed with the low-affinity dye, reflecting the sensitivity to low Ca^{2+} concentrations. However, there was still no difference between depolarization-preconditioned neurons and control-preconditioned neurons ($t_{1/2} = 17.4 \pm 3.2$ s for control cells, $t_{1/2} = 15.1 \pm 2.2$ s for depolarization preconditioned cells, $p > 0.05$, $n = 18$ and 21 cells per respective condition).

In a final test of whether preconditioning recruited postsynaptic adaptations, we evaluated the effect of depolarization preconditioning on toxicity induced by exogenous glutamate. This experiment exploited the pivotal role of glutamate receptor overstimulation in hypoxic cell death (Choi and Rothman, 1990). Direct overstimulation of postsynaptic receptors by-passes endogenous glutamate release contributing to hypoxic damage. Thus, if depolarization preconditioning acts through presynaptic

mechanisms, it should be ineffective against exogenous glutamate toxicity. If, however, preconditioning works through postsynaptic mechanisms, it should retain effectiveness and protect against direct glutamate excitotoxicity. We found that depolarization preconditioning was not effective in protecting neurons against exogenously applied glutamate (Fig. 3). In control cultures preconditioned with 30 mM NaCl, application of 10 μ M glutamate for 5 min killed over 60% of neurons, evaluated 24 h post-insult (Fig. 3). This was indistinguishable from cell loss in cultures preconditioned with depolarizing KCl (Fig. 3). Glutamate-induced cell loss following control preconditioning was similar to or slightly milder than that achieved in our hypoxia model. This result strongly suggests that the neuroprotection from hypoxia by strong depolarization preconditioning is of presynaptic origin, consistent with a potential role for presynaptic muting.

Preconditioning protection extends to oxygen/glucose deprivation:

Fig. 1 shows that depolarization preconditioning protects against a 2.5 h hypoxic insult, which normally kills approximately 70% of neurons in our paradigm. Previous studies in cortical neurons have shown that deprivation of both oxygen and glucose may be a more severe insult than hypoxia alone (Goldberg and Choi, 1993). We wanted to test whether this potentially more severe insult is also attenuated by presynaptic muting induced by depolarization preconditioning. As in the hypoxia paradigm we exposed cells to 4 h of high KCl (30 mM) or 4 h of a non-depolarizing NaCl control. After preconditioning, the neurons were removed from the preconditioning stimulus and immediately subjected to OGD for 2.5 h. The cells were then returned to their original

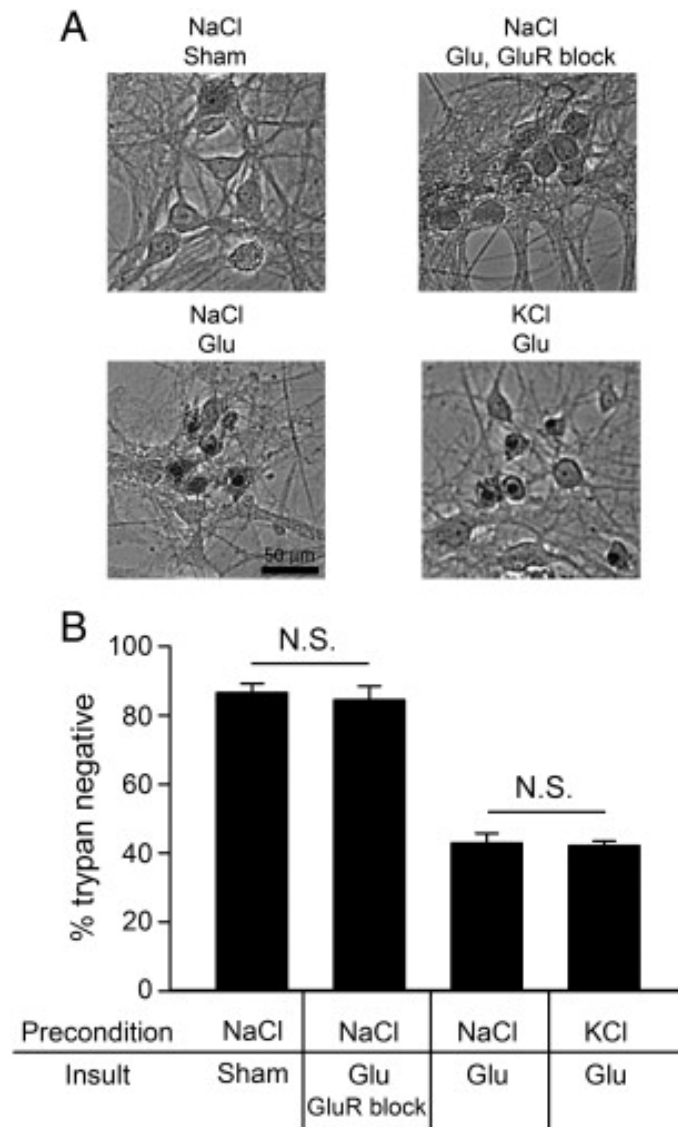


Figure 3. Exogenous glutamate toxicity is unaffected by depolarization preconditioning. **A.** Brightfield photomicrographs of trypan blue-stained fields from the indicated conditions in a single experiment. Exogenous glutamate (Glu; 10 μ M for 5 min) replaced hypoxia in the preconditioning/insult paradigm. Upper left: control cells 24 h post-preconditioning with 30 mM NaCl and sham glutamate exposure that included media exchange. Upper right: protection control cells preconditioned with 30 mM NaCl, subjected to 10 μ M glutamate (5 min) with ionotropic GluR antagonists present (1 μ M NBQX + 100 μ M D-APV). Lower left: cells preconditioned with 30 mM NaCl, subjected to 10 μ M glutamate for 5 min. Lower right: cells preconditioned with 30 mM KCl, then subjected to 5 min of 10 μ M glutamate. **B.** Summary graph of the conditions shown in A ($n = 5$). Glutamate significantly increased trypan blue staining compared with the NaCl sham condition in this dataset ($p < 0.05$, Bonferroni corrected t -test). However, depolarization preconditioning did not significantly alter the severity of cell loss. As with hypoxia, GluR block (1 μ M NBQX, 100 μ M D-APV) effectively protected neurons from glutamate-induced death, consistent with the pivotal role of excitotoxicity in both insults.

media and were allowed to incubate under normal conditions for 24 h, after which we performed trypan blue staining. As expected, OGD for 2.5 h killed more cells than hypoxia alone (compare Fig. 4 and Fig. 1). Nevertheless, as with the milder insult, depolarization preconditioning significantly protected neurons from the more severe OGD insult (Fig. 4).

Depolarization preconditioning protection does not involve GABA_A receptor activation:

Previous work has suggested that a primary mechanism by which cortical neurons are desensitized to hypoxic damage after a depolarizing preconditioning stimulus is through enhancement of GABA_A receptor activity during subsequent lethal ischemia (Grabb et al., 2002). On the other hand, some studies have suggested a more limited contribution of GABA_A receptor activation in other forms of preconditioning (Lange-Asschenfeldt et al., 2005). Studies conducted in our lab have shown that muting is induced at glutamate synapses, but not GABA synapses, by strong depolarization (Moulder et al., 2004). Therefore, if we are correct that glutamate presynaptic muting is responsible for neuroprotection in our preconditioning paradigm, we expect no role for GABA in the protection. To test whether neuroprotection in our paradigm is dependent on GABA_A receptor activity during hypoxia (Grabb et al., 2002), we preconditioned neurons, followed by blockade of GABA_A activity during the insult. The non-competitive GABA_A receptor antagonist picrotoxin (100 μM) was used to circumvent the possibility that competitive antagonists might be overwhelmed by endogenous GABA release. Blockade of GABA_A receptors during hypoxia did not affect cell survival in our paradigm (Fig. 5A), and picrotoxin did not affect the protection afforded by KCl

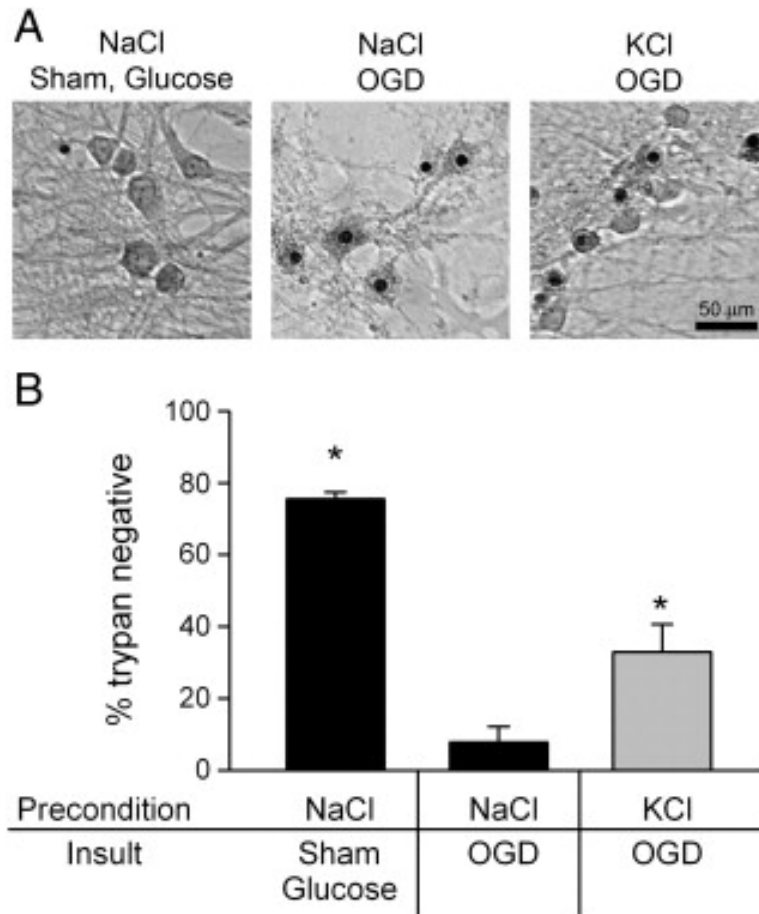


Figure 4. Depolarization preconditioning protects against oxygen-glucose deprivation (OGD). **A.** Brightfield micrographs of trypan blue staining from each condition: (left to right) control dish 24 h post-preconditioning with 30 mM NaCl and sham OGD; dish preconditioned with 30 mM NaCl, subjected to OGD for 2.5 h; dish preconditioned with 30 mM KCl, then subjected to OGD for 2.5 h. **B.** Summary graph showing protection afforded by KCl depolarization preconditioning ($n = 4$). Asterisks denote $p < 0.05$ compared with the NaCl OGD condition (Bonferroni corrected t -tests). Gray bar emphasizes the major hypothesized result of KCl preconditioning protection.

preconditioning (Fig. 5A). These results support the idea that reduction in presynaptic glutamate release, rather than altered GABA signaling, is key to the preconditioning protection from strong depolarization.

Depolarization preconditioning protection does not involve A1 receptors:

A1 adenosine receptor activation may contribute to ischemic preconditioning tolerance (Nakamura et al., 2002; and Perez-Pinzon et al., 1996) and selectively affect glutamate transmission in the hippocampus (Yoon and Rothman, 1991). Blockade of A1 adenosine receptors, however, does not abolish depolarization-induced presynaptic muting of glutamate terminals by high KCl exposure (Crawford et al., 2011). To distinguish depolarization-induced muting from adenosine-dependent forms of preconditioning, we co-administered the A1 adenosine receptor blocker 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; 200 nM) during depolarization preconditioning. As before, the cells were subsequently challenged with 2.5 h of hypoxia. The neuroprotective effects of elevated KCl were not altered by A1 receptor inhibition (Fig. 5B). This suggests that in our paradigm, unlike other forms of preconditioning (Nakamura et al., 2002; and Perez-Pinzon et al., 1996), the protection from hypoxia by presynaptic silencing of glutamate release is unlikely to involve A1 adenosine receptor activation during preconditioning.

Depolarization preconditioning protection from hypoxia does not require Ca²⁺ influx:

A unique aspect of the induction of presynaptic silencing by strong depolarization is that it is Ca²⁺-independent, unlike most other forms of synaptic plasticity (Crawford et

al., 2011). Therefore, as an additional test of the involvement of presynaptic muting in preconditioning protection, we preconditioned cells with KCl in the absence of extracellular Ca^{2+} (plus 0.1 mM EGTA to chelate residual Ca^{2+} ; Fig. 5C). Removing Ca^{2+} did not affect the ability of depolarization preconditioning to protect from hypoxic excitotoxicity (compare Fig. 5C with Fig. 1C). These results demonstrate a signature feature of presynaptic muting and are additional support for the idea that muting plays a strong role in preconditioning protection. Furthermore, the finding excludes Ca^{2+} -dependent secretion of neurotransmitters and neuromodulators during preconditioning in the protection.

Proteasome inhibition prevents depolarization preconditioning protection:

The UPS is involved in the regulation of synaptic function, including presynaptic development and function (Haas and Broadie, 2008; Hegde and DiAntonio, 2002; and Willeumier et al., 2006). Recently a postsynaptic UPS-dependent mechanism induced by N-Methyl-D-aspartic acid (NMDA) receptor activation was implicated in rapid ischemic tolerance, a neuroprotective effect of subtoxic conditioning ischemia (Meller et al., 2008). Proteasome inhibition also prevents the induction of presynaptic silencing (Jiang et al., 2010). If presynaptic muting is involved in depolarization preconditioning-induced neuroprotection, proteasome inhibition should reverse the protection from depolarization preconditioning. To investigate the role of the UPS in preconditioning neuroprotection, we co-applied the proteasome inhibitor MG-132 (3 μM) during the preconditioning period of high KCl (30 mM) exposure. Protection from hypoxia after the high KCl preconditioning period was completely abolished by proteasome inhibition during

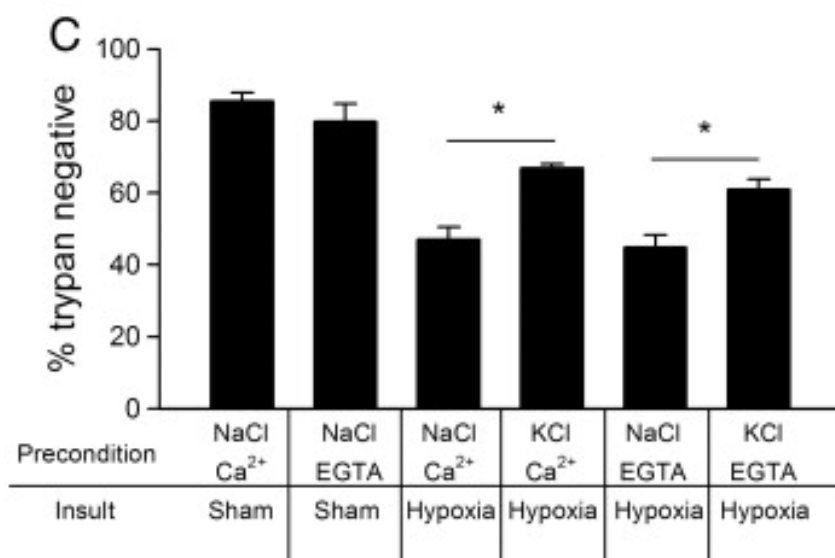
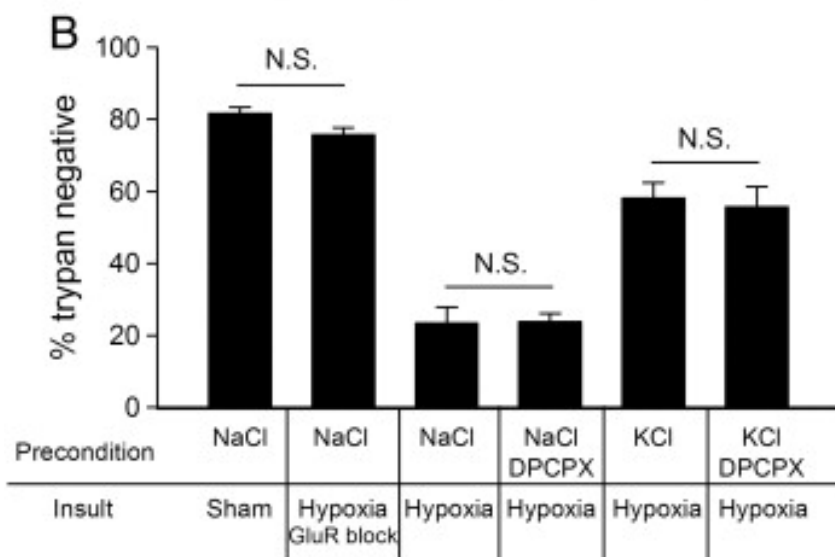
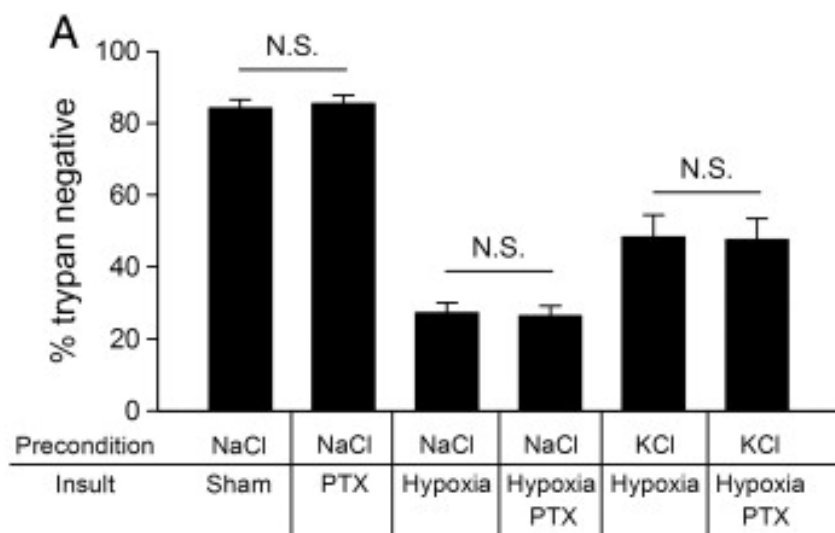


Figure 5. Depolarization preconditioning protection from hypoxia does not depend upon GABA_A receptor modulation, adenosine A1 receptor activation, or extracellular Ca²⁺ influx. **A.** The non-competitive GABA_A antagonist picrotoxin (PTX; 100 μM), applied during hypoxia (2.5 h), did not prevent depolarization protection. Summary of the indicated experimental conditions (*n* = 6). ‘N.S.’ indicates lack of significant difference in comparisons of the effect of PTX with the corresponding condition in the absence of PTX. **B.** The A1 receptor antagonists DPCPX (200 nM) did not block depolarization preconditioning protection. Summary of various 4 h preconditioning conditions. Hypoxic insult was 2.5 h (*n* = 5). ‘N.S.’ indicates a lack of difference for the indicated comparisons. **C.** Summary graph showing protection afforded by KCl depolarization preconditioning (*n* = 5) independent of the addition of 1.8 mM extracellular Ca²⁺ to the Ca²⁺ free conditioning media. For all panels, neuronal survival was assessed 24 h after insult with trypan blue exclusion. Asterisks denote *p* < 0.05 comparisons (Bonferroni corrected *t*-tests).

preconditioning (Fig. 6A). Taken together with preceding evidence (Fig. 1, Fig. 2 and Fig. 3) for a presynaptic locus of KCl neuroprotection, this result is consistent with the idea that UPS-dependent presynaptic silencing induced prior to the insult protects neurons from hypoxia. Because D-APV and NBQX were present in preconditioning solutions, it is unlikely that MG-132 acts through these postsynaptic receptor systems to reverse the presynaptic protection afforded by depolarization. Furthermore, when we bypassed presynaptic terminals and killed neurons by direct exogenous glutamate exposure, there was no difference in survival following control preconditioning, KCl preconditioning, MG-132 preconditioning, or a combination of KCl and MG-132 preconditioning (Fig. 6B). Although the effects on the UPS from hypoxia are likely complex and operate in both presynaptic and postsynaptic compartments, our results suggest that MG-132 reverses protection in our model by a presynaptic mechanism induced during preconditioning, upstream of postsynaptic receptor overstimulation. Control experiments also verified that neither KCl nor MG-132 affected cell survival in the absence of insult. A 4 h application of 30 mM KCl or 3 μ M MG-132 alone was not toxic to the neurons (Fig. 6C).

Hypoxia induces muting:

The preceding results establish that, in principle, muting of presynaptic glutamate release induced prior to insult is capable of protecting neurons from subsequent hypoxic/OGD excitotoxicity, but do neurons employ muting of glutamate synapses during the insult itself? Depolarization of neurons is an important early consequence of hypoxic/ischemic insult and is reinforced by subsequent glutamate release and receptor

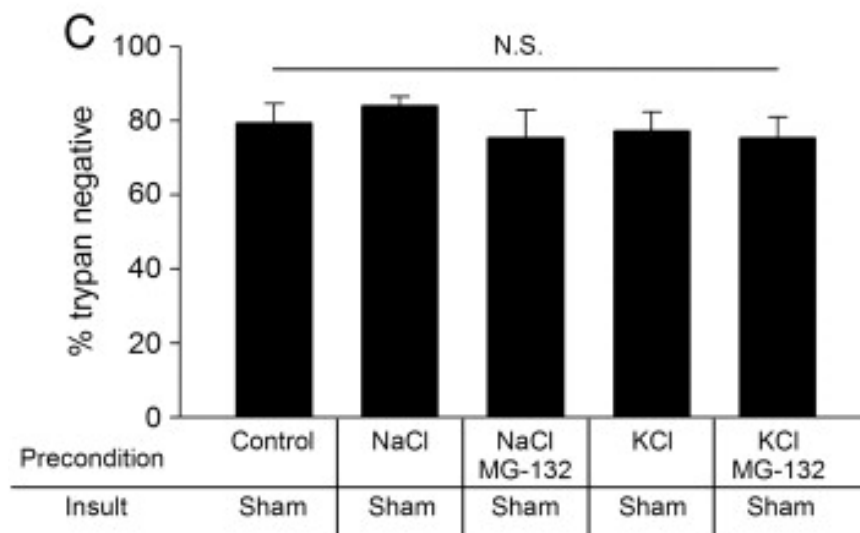
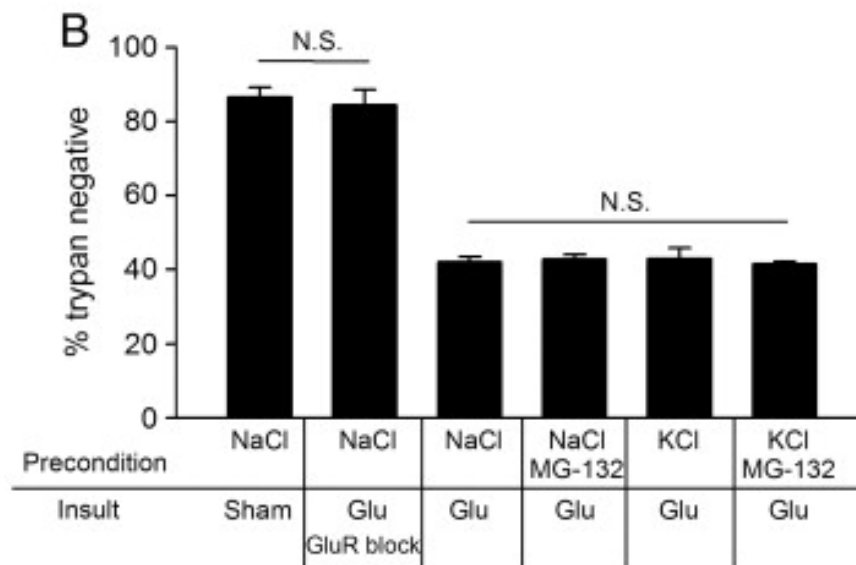
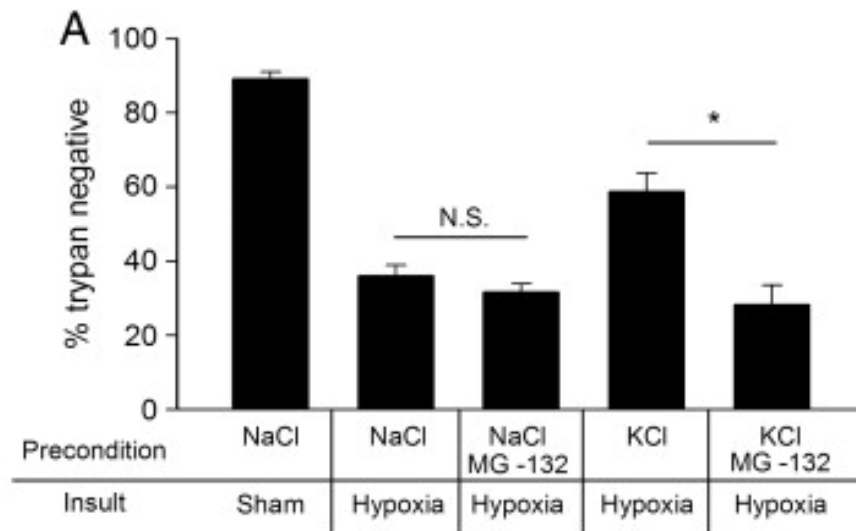


Figure 6. Proteasome inhibition prevents depolarization preconditioning protection through a presynaptic mechanism. **A.** Summary of neuronal survival after several 4 h preconditioning conditions and 2.5 h hypoxic exposure ($n = 5$). MG-132 (3 μM , co-applied for 4 h with 30 mM KCl) prevented the protective effect of depolarization. Indicated comparisons represent the major hypothesized effects (asterisk denotes $p < 0.05$ with Bonferroni correction). Other comparisons that showed a significant difference from insult alone (NaCl hypoxia) were the NaCl sham control and the KCl hypoxia conditions. There was no difference between the NaCl hypoxia condition and NaCl hypoxia plus MG-132. **B.** Summary of cell survival after indicated preconditioning conditions followed by an insult of exogenously applied glutamate (Glu; 10 μM for 5 min). There was no effect of KCl or of MG-132 on cell survival using the glutamate insult ($n = 5$), suggesting a presynaptic mechanism of depolarization preconditioning protection. No comparison of the NaCl/glutamate group with any of the other indicated groups yielded a significant difference. Glutamate treatment caused significant death relative to control ($p < 0.05$, Bonferroni corrected t -test for multiple comparisons). **C.** Control experiment testing direct effects of KCl and MG-132 on cell survival during various 4 h preconditioning paradigms in the absence of hypoxia ($n = 5$). No comparison with the control sham condition exhibited a significant difference (unpaired t -tests vs. control). For all experiments depicted in panels A, B, and C neuronal survival was evaluated with trypan blue 24 h after the insult.

activation (Moskowitz et al., 2010). If depolarization is rapid and strong enough to induce muting, presynaptic silencing may be an important endogenous regulator of damage threshold. To investigate this, we used FM1-43fx uptake to assess presynaptic function immediately after a 2 h hypoxic event, without any preceding preconditioning. As shown in Fig. 2, muted glutamate terminals immunolabel with an antibody against the vesicular glutamate transporter 1 (vGluT-1) but not with the activity-dependent FM dye (Moulder et al., 2004). Hypoxia exposure clearly increased the number of mute synapses (Fig. 7). The muting induced by hypoxia was completely prevented by the inclusion of MG-132 (3 μ M) during the hypoxic challenge (Fig. 7B), suggesting that hypoxia and depolarization activate the same proteasome-dependent signaling cascade. As previously observed (Crawford et al., 2011; and Jiang et al., 2010), MG-132 had no effect on the basal percentage of silent terminals (Fig. 7B). Further, we failed to find a significant effect of MG-132 on overall glutamate synapse density (97.8 ± 6.5 vGluT-1 positive synapses per field in control, 117.0 ± 17.2 puncta mm^2 after MG-132 treatment, $n = 5$ experiments, $p > 0.3$). Finally, in two previously published papers by our group, we failed to detect any effect of MG-132 alone on excitatory postsynaptic currents (Crawford et al., 2011; and Jiang et al., 2010). These results offer direct evidence that proteasome-dependent presynaptic silencing occurs during hypoxia and suggest that presynaptic muting is an endogenous cellular defense mechanism that may reduce damage even without prior preconditioning designed to invoke synaptic muting.

Proteasome inhibition exacerbates neuronal death during a hypoxic insult:

To test directly whether proteasome-dependent presynaptic muting observed in

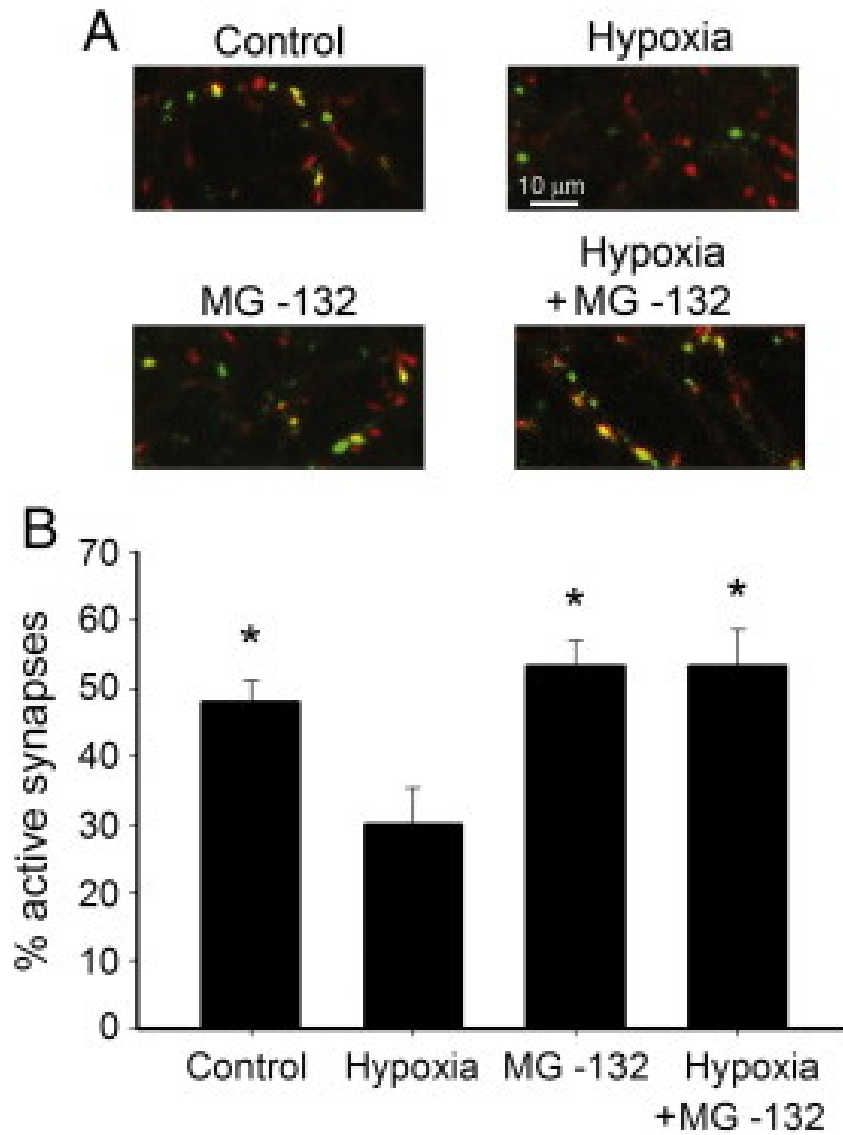


Figure 7. Hypoxia induces proteasome-dependent presynaptic silencing. **A.** FM1-43fx/vGluT-1 correspondence assay. Green = FM1-43fx. Red = vGluT-1. Red puncta with no green overlap are mute synapses, while yellow indicates overlap and active synapses. A 2 h preconditioning period with or without MG-132 (3 μ M) was followed by a 2 h hypoxic insult (or sham), then immediately by the FM1-43fx assay. **B.** Summary of experiments depicted in panel A showing the percentage of active synapses after hypoxic insult with and without MG-132 co-incubation ($n = 25$ fields from 5 independent experiments). Asterisk denotes $p < 0.05$ compared with hypoxia alone (Bonferroni corrected t -tests). Comparisons of control versus MG-132, control versus hypoxia plus MG-132, and MG-132 versus hypoxia plus MG-132 showed no differences.

Fig. 7 reduces the damage wrought by a moderate hypoxic insult, we tested the effect of proteasome inhibition on neuronal loss induced by hypoxia. We co-applied MG-132 during a 2 h hypoxic event (Fig. 8A). We chose an insult that would provide approximately 50% neuronal death in order to ensure detection of either protection or exacerbation of cell death. MG-132 during hypoxia significantly increased neuronal death (Fig. 8B). Again, MG-132 did not affect neuronal death when applied to neurons for a total of 4 h without hypoxia (Figs. 6C and 8B). Further, we found that MG-132 preincubation, followed by co-incubation with 10 μ M glutamate to bypass presynaptic effects, did not alter direct, glutamate induced death (Fig. 8C). These results are consistent with a presynaptic action of MG-132. Taken together, the results of Fig. 7 and Fig. 8 suggest that presynaptic silencing raises the threshold for neurotoxicity during a depolarizing insult.

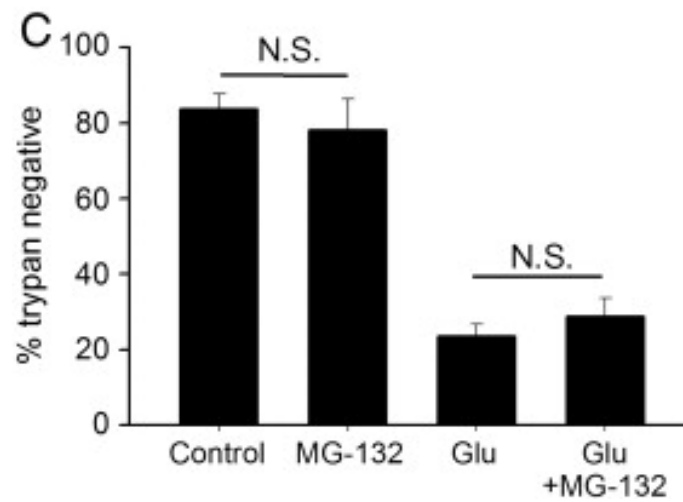
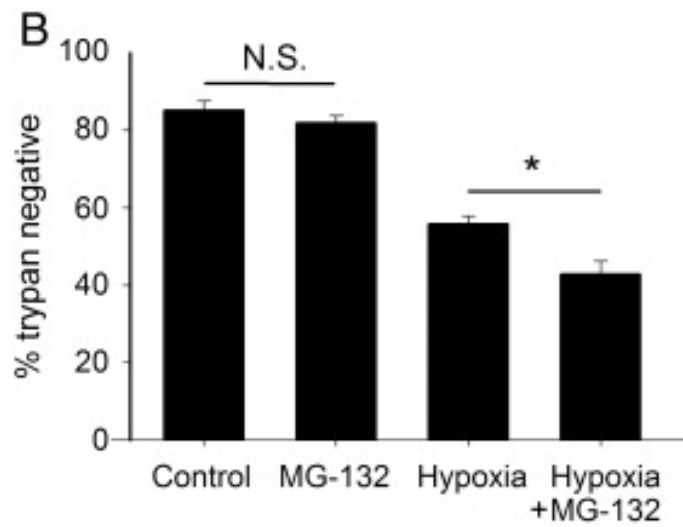
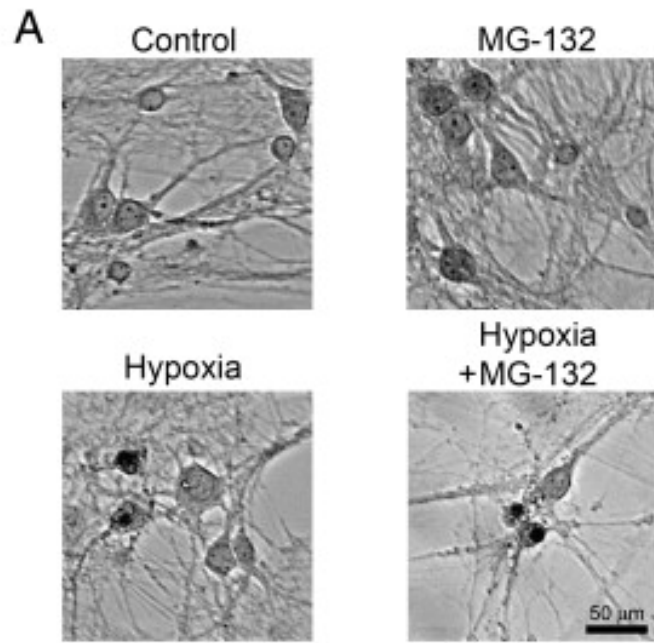


Figure 8. Proteasome inhibition during hypoxia exacerbates neuronal damage. A. Brightfield trypan blue-stained fields from a representative experiment. Neurons were incubated with or without MG-132 for 2 h to allow drug penetration, then subsequently co-incubated with MG-132 for additional 2 h with or without hypoxia. Culture media was then exchanged, and cells were incubated normally for 24 h after which trypan blue staining was performed. Upper left: control with no MG-132 or hypoxia. Upper right: 4 h total MG-132 exposure (no hypoxia). Lower left: 2 h hypoxia alone. Lower right: 2 h MG-132 followed by hypoxia/MG-132 co-incubation for 2 h (4 h total exposure to MG-132). **B.** Summary of A showing that MG-132 exacerbates hypoxia-induced death ($n = 7$). Asterisk denotes $p < 0.05$ (Bonferroni corrected t -test). **C.** Summary of control for postsynaptic effects of MG-132 ($n = 5$ experiments). 10 μ M glutamate (Glu) treatment was used as a surrogate for hypoxic insult. There was no significant exacerbation of glutamate-induced damage by MG-132. As with hypoxia experiments in B, cells were pre-incubated in MG-132 for 2 h prior to insult.

Discussion

We have shown that a form of synaptic plasticity working through a presynaptic UPS-dependent mechanism significantly protects neurons from *in vitro* hypoxic/ischemic insults. Importantly, muting is endogenously invoked by hypoxia with no prior conditioning to limit the damage severity inflicted during a moderate insult. Protection is achieved by invoking an endogenous mechanism that mutes vesicular glutamate release at presynaptic terminals. Among a growing body of research into preconditioning and tolerance (Gidday, 2006; and Sapolsky, 2001), presynaptic muting is a previously unknown UPS-dependent mechanism of tolerance. Because muting is part of a native cellular defense mechanism in response to stressful depolarization, it might be a good target for future therapeutics. Our insights into this UPS-dependent mechanism demonstrate presynaptic regulation of neuronal homeostasis and suggest new approaches that may benefit disorders involving glutamatergic synaptic dysfunction.

Presynaptic muting appears particularly well suited as an adaptation against strong insults like hypoxia/ischemia. Strong depolarization and associated sustained Ca^{2+} influx during these insults are likely to quickly overwhelm many presynaptic adaptive mechanisms, such as G-protein-mediated decreases in presynaptic Ca^{2+} influx (Brown and Sihra, 2008). Because muting effectively eliminates vesicle release competence to Ca^{2+} and other secretagogues (Moulder et al., 2006; and Moulder et al., 2004), muting should be especially effective at disrupting the cycle of pathological depolarization during the insult. On the other hand, we acknowledge that strategies designed to disrupt glutamate signaling have to date proved disappointing in clinical settings (Gidday, 2006; Lipton, 2007; and Moskowitz et al., 2010), and presynaptic muting may be ineffective

during strong insults that recruit non-synaptic glutamate release (Rossi et al., 2000).

Proteasome inhibition exacerbated damage and also inhibited presynaptic silencing in response to hypoxic insult (Fig. 7 and Fig. 8). Proteasome activity is increased during strong depolarization, and proteasome inhibition also prevents presynaptic silencing during strong depolarization (Jiang et al., 2010). In the present study, proteasome inhibition prevented the neuronal protection from strong depolarization followed by hypoxia (Fig. 6A). Similar exacerbation of cell death was recently described in an ischemia model, where an NMDA receptor and UPS-dependent postsynaptic remodeling mechanism was implicated in a rapid ischemic tolerance (Meller et al., 2008). These previous results fit with known UPS roles in the postsynaptic compartment (Mabb and Ehlers, 2010; and Tai and Schuman, 2008). On the other hand, the UPS is also important for aspects of presynaptic development and function (DiAntonio and Hicke, 2004; Jiang et al., 2010; and Willeumier et al., 2006). Our observations fit more closely with this latter literature. Postsynaptic NMDA receptor activation is not required for our preconditioning protection, as D-APV was present in all preconditioning treatments in our experiments to block NMDA receptor-dependent forms of plasticity. Furthermore, a postsynaptic mechanism of protection is excluded by our experiments examining preconditioning effects on exogenous glutamate damage and postsynaptic Ca^{2+} handling in response to exogenously applied glutamate after preconditioning (Fig. 2, Fig. 3 and Fig. 6).

Our experiments were designed to isolate presynaptic contributions to self-defense mechanisms and therefore do not negate contributions of previously described postsynaptic mechanisms of adaptive neuroprotection under some conditions. These

mechanisms may be most important in the most severe insults where non-synaptic glutamate release is recruited (Rossi et al., 2000). Further work is needed to elucidate the relative importance of presynaptic silencing and other adaptive, self protective mechanisms.

Although UPS-dependent neuroprotection in our model is mainly presynaptic and distinct from previously described UPS-dependent and NMDA-dependent forms of tolerance (Grabb and Choi, 1999), it is possible that complex changes in UPS function accompany insult. Although proteasome inhibition has been reported following brain ischemia and is associated with ATP depletion (Asai et al., 2002; and Thompson et al., 2008), this does not exclude local persistence of or increases in UPS activity in certain tissue compartments, specific cell types, or specific subcellular compartments. We expect that UPS-dependent presynaptic muting will be particularly relevant to glutamate terminals in the penumbra of an insult where a certain degree of neuronal function is preserved.

Our results demonstrating a role of the UPS in presynaptic muting and cell survival (Fig. 7 and Fig. 8) also exclude a possible alternative explanation for the muting observed in response to hypoxia: cell death. Although cell loss was not evident for several hours after the hypoxic insult and there was little evidence of presynaptic damage at the time of synaptic imaging (Fig. 7), the presynaptic silencing observed might be a trivial result of dying neurons rather than a neuroprotective response of viable neurons. The effect of MG-132 clearly argues against this possibility. MG-132 application during hypoxia prevented muting (Fig. 7) but exacerbated neuronal damage (Fig. 8). This pattern of results excludes damage as a cause of muting and strongly suggests that presynaptic

function is an important determinant of subsequent cell loss.

Additional study of the pathways responsible for presynaptic muting may guide strategies for therapeutic exploitation of these pathways. Upstream targets may include pertussis toxin-sensitive $G_{i/o}$ -linked G-protein receptors (Crawford et al., 2011). Downstream effectors may include relevant targets of the UPS (Jiang et al., 2010). Depolarization-induced muting results in decreased levels of the vesicle priming proteins Rim1 α and Munc13-1, and overexpression of Rim1 α prevents presynaptic silencing (Jiang et al., 2010). However, the proteasome also potentially regulates a host of other synaptic proteins such as SNAP-25, syntaxin, and synaptophysin (Chin et al., 2002; Ma et al., 2005; Wheeler et al., 2002; and Willeumier et al., 2006) among many other candidates, befitting the ubiquitous nature of the UPS system. Targeting the specific proteins responsible for muting may offer a viable, selective therapeutic strategy.

With these results we provide new insight into a homeostatic neuronal defense mechanism at glutamatergic presynaptic terminals. Pharmacological exploitation of a mechanism reducing presynaptic glutamate release could potentially treat excitotoxic damage resulting from stroke, neonatal hypoxia, epilepsy, and head trauma at the time of injury, where treatment options are quite limited and postsynaptic interventions have failed. Presynaptic muting appears to render presynaptic terminals incompetent to exocytose even when challenged with sustained Ca^{2+} rises. The mechanism we report here does not require lengthy latent periods for its induction and is immediately available as a homeostatic mechanism. Additional study of presynaptic muting will increase our basic understanding of hippocampal synaptic function. Further study may also identify potential treatment targets related to muting that could benefit various neurological and

psychiatric disorders involving glutamate dysfunction.

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