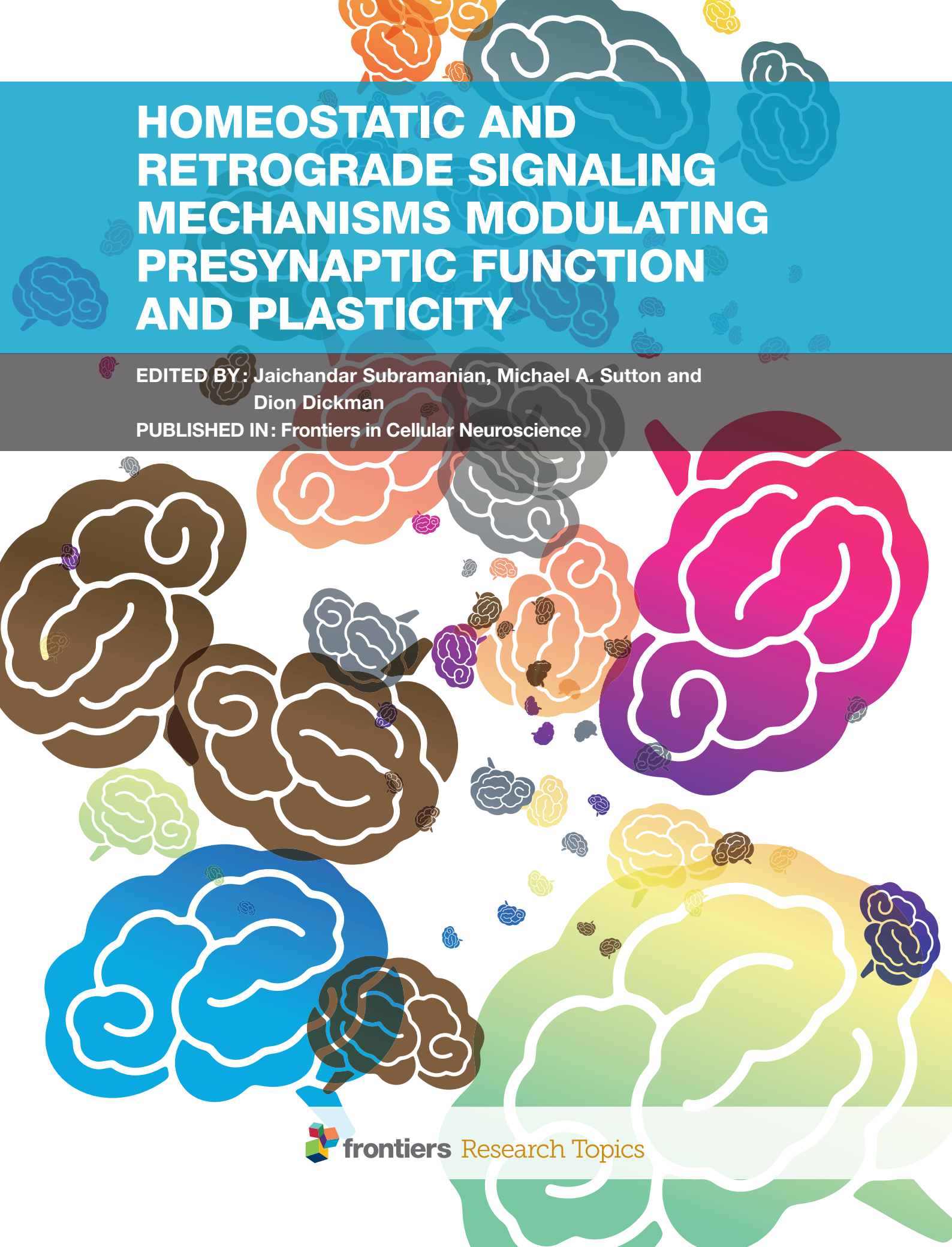
HOMEOSTATIC AND RETROGRADE SIGNALING MECHANISMS MODULATING PRESYNAPTIC FUNCTION AND PLASTICITY

EDITED BY: Jaichandar Subramanian, Michael A. Sutton and Dion Dickman

PUBLISHED IN: Frontiers in Cellular Neuroscience



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ISSN 1664-8714
ISBN 978-2-88919-704-0
DOI 10.3389/978-2-88919-704-0

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HOMEOSTATIC AND RETROGRADE SIGNALING MECHANISMS MODULATING PRESYNAPTIC FUNCTION AND PLASTICITY

Topic Editors:
Jaichandar Subramanian, Massachusetts Institute of Technology, USA
Michael A. Sutton, University of Michigan, USA
Dion Dickman, University of Southern California, USA

Activity within neural circuits shapes the synaptic properties of component neurons in a manner that maintains stable excitatory drive, a process referred to as homeostatic plasticity. These potent and adaptive mechanisms have been demonstrated to modulate activity at the level of an individual neuron, synapse, circuit, or entire network, and dysregulation at some or all of these levels may contribute to neuropsychiatric disorders, intellectual disability, and epilepsy. Greater mechanistic understanding of homeostatic plasticity will provide key insights into the etiology of these disorders, which may result from network instability and synaptic dysfunction. Over the past 15 years, the molecular mechanisms of this form of plasticity have been intensely studied in various model organisms, including invertebrates and vertebrates. Though once thought to have a predominantly postsynaptic basis, emerging evidence suggests that homeostatic mechanisms act on both sides of the synapse through mechanisms such as retrograde signaling, to orchestrate compensatory adaptations that maintain stable network function. These trans-synaptic signaling systems ultimately alter neurotransmitter release probability by a variety of mechanisms including changes in vesicle pool size and calcium influx. These adaptations are not expected to occur homogenously at all terminals of a pre-synaptic neuron, as they might synapse with neurons in non-overlapping circuits. However, the factors that govern the homeostatic control of synapse-specific plasticity are only beginning to be understood. In addition to our limited molecular understanding of pre-synaptic homeostatic plasticity, very little is known about its prevalence in vivo or its physiological and disease relevance. In this research topic, we aim to fill the aforementioned void by covering a broad range of topics that include:

- Identification of signaling pathways and mechanisms that operate globally or locally to induce specific pre-synaptic adaptations.
- The nature of pre-synaptic ion channels relevant to this form of plasticity and their synapse-specific modulation and trafficking.
- Development and utilization of new tools or methods to study homeostatic plasticity in axons and pre-synaptic terminals
- Novel mechanisms of homeostatic adaptations in pre-synaptic neurons.
- Postsynaptic sensors of activity and retrograde synaptic signaling systems.
- A comprehensive analysis of the kinds of pre-synaptic adaptations in diverse neural circuits and cell types.
- Identification of physiological or developmental conditions that promote pre-synaptic homeostatic adaptations.

- How activity-dependent (Hebbian) and homeostatic synaptic changes are integrated to both permit sufficient flexibility and maintain stable activity.
- Relevance of pre-synaptic homeostatic plasticity to the etiology of neuropsychiatric disorders.
- Computational modeling of pre-synaptic homeostatic plasticity and network stability

Citation: Subramanian, J., Sutton, M. A., Dickman, D., eds. (2015). Homeostatic and Retrograde Signaling Mechanisms Modulating Presynaptic Function and Plasticity. Lausanne: Frontiers Media. doi: 10.3389/978-2-88919-704-0

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frontiers in Cellular Neuroscience

EDITORIAL
published: 30 September 2015
doi: 10.3389/fncel.2015.00380

Editorial: Homeostatic and retrograde signaling mechanisms modulating presynaptic function and plasticity

Jaichandar Subramanian[1]* *and Dion Dickman*[2]

[1] Picower Institute for Learning and Memory, Massachusetts Institute of Technology, Cambridge, MA, USA, [2] Department of Neurobiology, University of Southern California, Los Angeles, CA, USA

Keywords: homeostatic plasticity, presynaptic adaptation, retrograde signaling, neurotransmitter release, neurological disease

Dynamic reorganization of neural circuits can occur through the selective strengthening of synapses between neurons that are coactive in response to the encoded information (Shatz, 1990). However, the positive feedback resulting from synaptic strengthening and neuronal coactivity can lead to the destabilization of neuronal networks. Thus, synapses must maintain the capacity for change necessary for learning and memory, yet constrain this inherently destabilizing flexibility to enable stable neural function throughout life. Evidence has emerged in recent years that activity within neural circuits can shape the synaptic properties of component neurons in a manner that maintains stable excitatory drive, a process referred to as homeostatic synaptic plasticity (Turrigiano and Nelson, 2000; Pozo and Goda, 2010; Davis, 2013). Potent and adaptive homeostatic mechanisms have been demonstrated in a variety of systems to modulate activity at the level of an individual neuron, synapse, circuit, or entire network, and dysregulation at some or all of these levels may contribute to neuropsychiatric disorders, intellectual disability, and epilepsy (Wondolowski and Dickman, 2013). Greater mechanistic understanding of homeostatic plasticity will provide key insights into the etiology of these disorders, which may result from network instability and synaptic dysfunction. Over the past 15 years, the molecular mechanisms of this form of plasticity have been intensely studied in various model organisms, including invertebrates and vertebrates (Davis and Müller, 2015). Though, once thought to have a predominantly postsynaptic basis, emerging evidence suggests that homeostatic mechanisms act on both sides of the synapse, through mechanisms such as retrograde signaling, to orchestrate compensatory adaptations that maintain stable network function (Vitureira et al., 2012). These trans-synaptic signaling systems ultimately alter neurotransmitter release probability by a variety of mechanisms including changes in vesicle pool size and calcium influx (Davis and Müller, 2015). These adaptations are not expected to occur homogenously at all terminals of a pre-synaptic neuron, as they might synapse with neurons in non-overlapping circuits. However, the factors that govern the homeostatic control of synapse-specific plasticity are only beginning to be understood.

The homeostatic mechanisms that regulate post-synaptic strength has been extensively studied and reviewed. In contrast, relatively little attention has been paid to pre-synaptic homeostatic plasticity mechanisms and retrograde signaling. This research topic is intended to shine light on this very important topic and we start this with a comprehensive review of different homeostatic plasticity mechanisms that govern neurotransmitter release probability (Lazarevic et al., 2013). Homeostatic adaptations are not merely functional changes that affect synaptic strength but involve a whole array of structural modifications in both excitatory and inhibitory neurons. A broad review on this topic is presented by Yin and Yuan (2014). This is followed by an in depth review on axonal adaptations in inhibitory neurons by Frias and Wierenga (2013).

OPEN ACCESS

Edited and reviewed by:
Egidio D'Angelo, University of Pavia, Italy

***Correspondence:**
Jaichandar Subramanian
jaichandar@gmail.com

Received: *12 July 2015*
Accepted: *11 September 2015*
Published: *30 September 2015*

Citation:
Subramanian J and Dickman D (2015) Editorial: Homeostatic and retrograde signaling mechanisms modulating presynaptic function and plasticity. Front. Cell. Neurosci. 9:380. doi: 10.3389/fncel.2015.00380

Many forms of homeostatic plasticity, such as synaptic scaling, occur over a period of several hours to days. In contrast, activity dependent strengthening of synapses (commonly referred to as Hebbian plasticity) is thought to happen on shorter time scales. Computational analyses reveal that slow forms of homeostasis are inadequate to counter the runaway excitability caused by hebbian plasticity. The next two chapters report novel means for achieving rapid homeostasis in response to network activity. The paper by Delattre V et al. describes how the timing of network activity in relation to that of spikes can determine the direction of plasticity (Delattre et al., 2015). The paper by Faghihi and Moustafa shows how Hebbian plasticity and fast retrograde signaling can interact to generate highest efficiency to encode differences in input stimuli (Faghihi and Moustafa, 2015).

In order to adapt to activity perturbations, neurons should have the machinery to sense and respond in adaptive ways that will lead to the modification of appropriate pre-synaptic function. These signals can be intrasynaptic or trans-synaptic depending on their locus of production and action. The next two chapters will provide extensive reviews on the roles of a retrograde signaling molecule Nitric Oxide (Hardingham et al., 2013) and the role of voltage gated calcium channels Frank, 2014b) in mediating (Frank, 2014b) homeostatic adaptations. In the following chapter, Qiu et al., provide evidence for endocannabinoid receptors in generating long-term depression in Molecular layer I-Purkinjee cell synapses in the cerebellum (Bing et al., 2015).

Much of the progress in our understanding of the interplay of pre- and post-synaptic cells to maintain synaptic strength has come from detailed studies of the larval neuromuscular junction of *Drosophila melanogaster* (Frank, 2014a). At this model glutamatergic synapse, genetic or pharmacological perturbations to postsynaptic receptors initiate a robust homeostatic signaling system in which a retrograde signal induces a precise increase in presynaptic release that compensates for this perturbation, restoring normal muscle excitability. The speed, accessibility, and genetic tractability of the fly NMJ make this a particularly attractive model to study the homeostatic control of presynaptic release. Using this system, Ueda and Wu report the mechanisms by which postsynaptic excitability is kept constant despite an increase in pre-synaptic growth when the larvae are reared at high temperatures (Ueda and Wu, 2015). In order to harness the full potential of this system, Brusich et al. has developed a new genetic tool that has enabled them to identify different molecules needed for the long-term maintenance of homeostatic synaptic plasticity (Brusich et al., 2015).

The potential importance of homeostatic mechanisms to circuit stability has long been recognized. Recently, many neurological and neuropsychiatric disease susceptibility genes have been found to be important for different aspects of homeostatic signaling. Interestingly, many of these diseases are comorbid with epilepsy, a condition predicted to be a consequence of runaway excitation resulting from impaired negative feedback plasticity mechanisms. The final two chapters review important links between presynaptic homeostatic mechanisms and neurological disease. The minireview by Meier et al. focuses on the presynaptic mechanisms that may contribute to the etiology of epilepsy (Meier et al., 2014). We conclude this topic with an extensive review on neurological disease susceptibility genes and their role in homeostatic synaptic plasticity (Wondolowski and Dickman, 2013).

Acknowledgments

This work was supported by a grant from the National Institutes of Health (NS019546) and research fellowships from the Alfred P. Sloan, Ellison Medical, Whitehall, Klingenstein-Simons, and Mallinckrodt Foundations to DKD.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in
CELLULAR NEUROSCIENCE

REVIEW ARTICLE
published: 03 December 2013
doi: 10.3389/fncel.2013.00244

Molecular mechanisms driving homeostatic plasticity of neurotransmitter release

Vesna Lazarevic[1], Santosh Pothula[2], Maria Andres-Alonso[2] and Anna Fejtova[2,3]*

[1] *Department of Neurochemistry and Molecular Biology, Leibniz Institute for Neurobiology, Magdeburg, Germany*
[2] *Research Group Presynaptic Plasticity, Leibniz Institute for Neurobiology, Magdeburg, Germany*
[3] *Center for Behavioral Brain Sciences, Magdeburg, Germany*

Edited by:
Jaichandar Subramanian, Massachusetts Institute of Technology, USA

Reviewed by:
Susanne Schoch, University of Bonn Medical Center, Germany
Dion Dickman, University of Southern California, USA

***Correspondence:**
Anna Fejtova, Research Group Presynaptic Plasticity, Leibniz Institute for Neurobiology, Brenneckestraße 6, 39118 Magdeburg, Germany
e-mail: afejtova@lin-magdeburg.de

Homeostatic plasticity is a process by which neurons adapt to the overall network activity to keep their firing rates in a reasonable range. At the cellular level this kind of plasticity comprises modulation of cellular excitability and tuning of synaptic strength. In this review we concentrate on presynaptic homeostatic plasticity controlling the efficacy of neurotransmitter release from presynaptic boutons. While morphological and electrophysiological approaches were successful to describe homeostatic plasticity-induced changes in the presynaptic architecture and function, cellular and molecular mechanisms underlying those modifications remained largely unknown for a long time. We summarize the latest progress made in the understanding of homeostasis-induced regulation of different steps of the synaptic vesicle cycle and the molecular machineries involved in this process. We particularly focus on the role of presynaptic scaffolding proteins, which functionally and spatially organize synaptic vesicle clusters, neurotransmitter release sites and the associated endocytic machinery. These proteins turned out to be major presynaptic substrates for remodeling during homeostatic plasticity. Finally, we discuss cellular processes and signaling pathways acting during homeostatic molecular remodeling and their potential involvement in the maladaptive plasticity occurring in multiple neuropathologic conditions such as neurodegeneration, epilepsy and neuropsychiatric disorders.

Keywords: presynaptic homeostatic plasticity, probability of neurotransmitter release, presynaptic muting, cytomatrix at the active zone, ubiquitin proteasome system

Brain function is based on signal transmission between neurons assembled in complex networks. Structural and functional reorganization of these neuronal networks in processes generally termed neuronal plasticity underlie the cognitive performance of the brain including learning and memory. This plasticity is mediated by the modification of the signal processing within and between neurons of these networks. Associative or Hebbian plasticity induces changes in synaptic transmission, which are use-dependent and lead to reinforcement of active and weakening of inactive circuits. However, if acting repetitively Hebbian plasticity processes would lead to saturation or complete inactivation of synaptic function and in turn to functional destabilization of neuronal networks. Homeostatic plasticity acts to balance changes induced by Hebbian plasticity and ensures the maintenance of physiological network activity levels.

At the cellular level homeostatic plasticity comprises modulation of cellular excitability and tuning of synaptic strength by both pre- and postsynaptic mechanisms. Here, we focus on the discussion of homeostatic plasticity processes affecting neurotransmitter release from presynaptic boutons. The phenomenon of homeostatic adaptation of the presynaptic release machinery to the levels of ongoing activity was first described for the *Drosophila* neuromuscular junction (NMJ; Petersen et al., 1997; Davis et al., 1998; Davis and Goodman, 1998). Morphological and functional alterations of presynapses induced by global changes of network activity have also been reported in mammalian neurons more than a decade ago (Murthy et al., 2001). In the following years, a number of studies reported presynaptic homeostatic plasticity induced by various stimuli and using different experimental models, ranging from cultured dissociated neurons and cultured brain slices to intact animals (Bacci et al., 2001; Burrone et al., 2002; Desai et al., 2002; Moulder et al., 2004; Thiagarajan et al., 2005; Wierenga et al., 2006). Taken together, these studies revealed that presynaptic efficacy is elevated when levels of activity decrease, while neurotransmitter release at synapses is less efficient after overall increase of network activity.

Abbreviations: AC, adenylate cyclase; AMPA, alpha-amino-3-hydroxy-5-methyl-isoxasole-4-propionic acid; AZ, active zone; Bic, bicuculline; cAMP, cyclic adenosine monophosphate; CASK, calcium/calmodulin-dependent serine protein kinase; Cav, voltage-dependent calcium channel; CAZ, cytomatrix at the active zone; CDK5, cyclin-dependent kinase 5; CNB, calcineurin B; CREB, cAMP response element-binding protein; DVGLUT, *Drosophila* vesicular glutamate transporter; E/I, excitation/inhibition; EEG, electroencephalography; GABA, gamma-aminobutyric-acid; GAD67, glutamic acid decarboxylase 67; mEPSC, miniature excitatory postsynaptic current; mIPSC, miniature inhibitory postsynaptic current; Munc-13, mammalian homologues of the *C. elegans* unc-13 gene; NBQX, 1,2,3,4-tetrahydro-6-nitro-2, 3-dioxo[f]quinoxaline-7-sulfonamide disodium; NMJ, neuromuscular junction; PhTx, philantotoxin-433; PKA, protein kinase A; PKC, protein kinase C; Pr, release probability; RIM, Rab3-interactning molecule; RP, recycling pool; RRP, readily releasable pool; RtP, resting pool; SV, synaptic vesicle; SVP, synaptic vesicle pools; SV2A, synaptic vesicle glycoprotein 2A; TRP, total recycling pool; TTX, tetrodotoxin; UPS, ubiquitin-proteasome system; VAMP-2, vesicle-associated membrane protein 2; VGAT, vesicular GABA transporter; VGLUT, vesicular glutamate transporter; VIAAT, vesicular inhibitory amino acid transporter.

Before describing how modulation of presynaptic efficacy occurs we briefly summarize the process of presynaptic neurotransmitter release. Neurotransmitter is stored in synaptic vesicles (SVs), which can release their content by controlled fusion with a specialized region of the presynaptic membrane named active zone (AZ). Central synapses contain around 200 SVs, which are not uniform regarding their functionality and localization. Different pools of vesicles have been described: the readily releasable pool (RRP, 5–9 vesicles), morphologically characterized by their physical contact with the AZ membrane, the recycling pool (RP) varies between 30 and 70% of all vesicles and contains SVs that can undergo exocytosis upon stimulation and resting pool (RtP) comprising vesicles that are incapable of exocytosis under physiological conditions (**Figure 1A**). Vesicles of the RRP are released within a few milliseconds to seconds during stimulation at 10–40 Hz (Stevens and Williams, 2007) or by the application of a hypertonic pulse of sucrose (Rosenmund and Stevens, 1996). Vesicles of RP are released upon prolonged stimulations, when RRP has been depleted. Together, RRP and RP form the total recycling pool (TRP). Vesicles of the RRP have the highest fusion probability of all vesicles. Therefore, the size of RRP is decisive for the synaptic release probability (Pr) and often assessed as a parameter of presynaptic strength (for comprehensive review on synaptic vesicle pools, SVP see Alabi and Tsien, 2012). Voltage-dependent calcium (Ca^{2+}) channels (Cavs), which open in response to action potential-driven depolarization of the presynaptic membrane and mediate the Ca^{2+} influx into boutons, are crucial for evoked release. On the one hand they are regulated with respect to their localization within the AZ (Gundelfinger and Fejtova, 2012; Sudhof, 2012) and on the other hand through modification of their properties by multiple signaling pathways (Catterall and Few, 2008). Mechanistically, homeostatic changes in presynaptic Pr were mostly attributed to the modulation of the SVP (especially of the RRP; Murthy et al., 2001; Moulder et al., 2006), and to changes in action potential-induced Ca^{2+} influx (Moulder et al., 2003; Frank et al., 2006; Zhao et al., 2011; Muller and Davis, 2012).

This review highlights the recent advances in our understanding of cellular mechanisms and identification of molecular players contributing to homeostatic modulation of the presynaptic Pr.

PRESYNAPTIC HOMEOSTATIC PLASTICITY IN *Drosophila*

A comprehensive review of homeostatic plasticity on *Drosophila* NMJ was published recently (Frank, 2013). In this section, we will

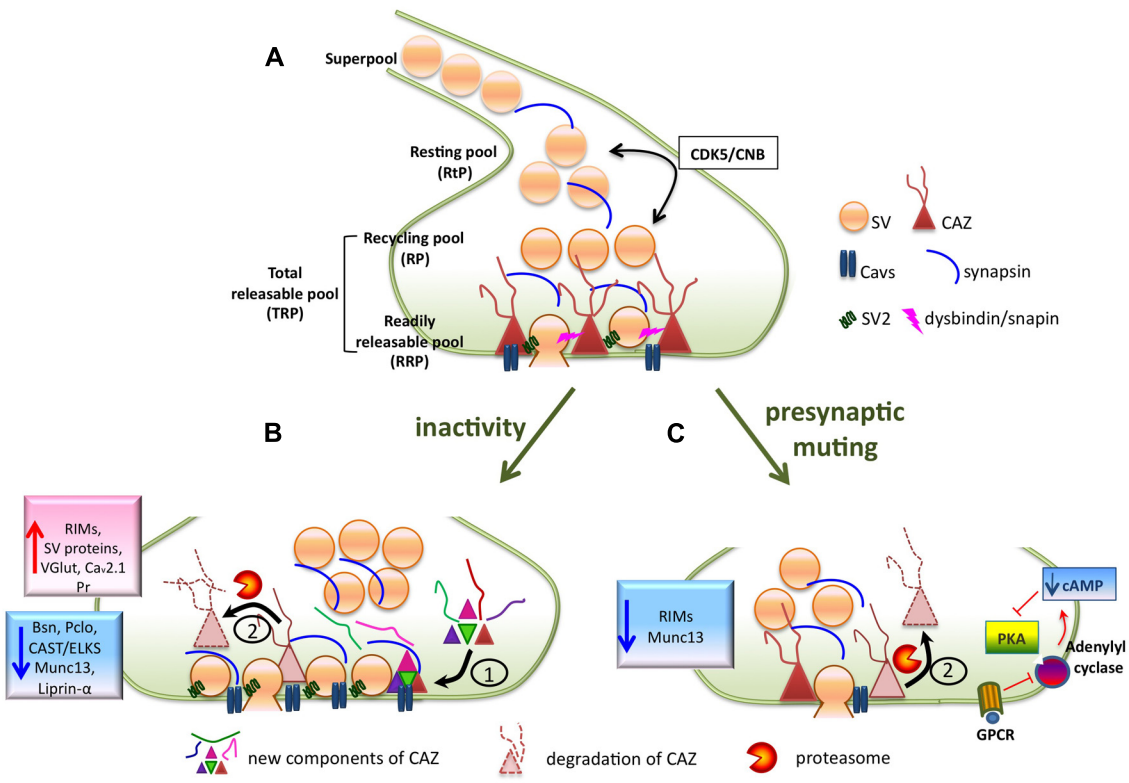

FIGURE 1 | (A) Scheme of a presynaptic bouton containing synaptic vesicles that can be assigned to functionally different pools (RRP, RP, RtP), the release machinery (depicted as cytomatrix at the active zone, CAZ) and the presynaptic Ca^{2+} channels Cavs. **(B)** Prolonged activity deprivation induces extensive structural (enlargement of active zones) and functional (increased RRP and Pr) remodeling of presynaptic active zones, which is associated with a molecular reorganization of the release machinery. Protein synthesis **(1)** as well as protein degradation via the ubiquitin-proteasome system (UPS), **(2)** play a role in this process. **(C)** Depolarization-induced presynaptic muting is characterized by decreased size of RRP, depressed Pr and the reduction of CAZ proteins RIM and Munc13, probably due to their degradation by UPS. Induction/recovery from presynaptic muting also involves regulation of adenylate cyclase activity, which is an upstream regulator of PKA.

summarize the homeostatic modulation of presynaptic function with emphasis on identified molecular mechanisms acting during the adaptation of neurotransmitter release in this model system.

Early observations on homeostasis-induced modifications of synaptic transmission at the NMJ arose from developmental studies. During *Drosophila* larval development the surface area of muscles increase dramatically in a short period of time, decreasing the input resistance and evoking a growth of presynaptic nerve terminals and an increment in the number of boutons and AZs, resembling homeostatic adaptations. Genetic manipulations leading to an increased muscle innervation results in a compensatory, target-specific decrease in presynaptic transmitter release, whereas decreased muscle innervation results in a compensatory increase in quantal size (Davis and Goodman, 1998). Similarly, when the functionality of glutamate receptors was genetically ablated or the postsynaptic membrane was hyperpolarized by expression of Kir2.1 potassium channel, the presynaptic terminal responded by increasing the quantal content (i.e., increasing the number of presynaptic vesicles released per stimulus; Petersen et al., 1997; Davis et al., 1998; Paradis et al., 2001). This is well comparable with the later described increase of Pr at synapses of the vertebrate central nervous system (Murthy et al., 2001), implying evolutionary conservation of homeostatic plasticity mechanisms.

In contrast to vertebrate synapses (Plomp et al., 1992; Han and Stevens, 2009), the manifestation of presynaptic homeostatic plasticity is remarkably rapid in *Drosophila* larvae. Potentiation of presynaptic release appeared within 5–10 min upon application of philantotoxin-433 (PhTx), a persistent use-dependent antagonist of glutamate receptors (Frank et al., 2006). Both rapid induction and persistent expression of presynaptic homeostatic plasticity were blocked by mutations of Cav2.1, the pore-forming subunit of *Drosophila* calcium channel encoded by the *cacophony (cac)* gene. A recent publication by the same group, revealed ephexin, a Rho-type guanine nucleotide exchange factor, as indispensable for homeostasis-evoked increase of neurotransmitter release (Frank et al., 2009). Ephexin acts primarily with Cdc42 in a signaling system that converges on Cav2.1, further supporting its key importance during presynaptic homeostatic plasticity. Of note, PhTx-induced presynaptic homeostatic plasticity does not require new protein synthesis as it was shown for some forms of presynaptic homeostatic plasticity in vertebrates (Sutton et al., 2006; Han and Stevens, 2009). Nevertheless, fast molecular remodeling of presynaptic release apparatus occurs during this rapid functional plasticity as demonstrated, e.g., for the protein bruchpilot (Weyhersmuller et al., 2011), which is enriched at presynaptic release sites upon pharmacological blockage of glutamate receptors. Bruchpilot is a core component of AZ specializations at the NMJ, named T-bars, implied in the organization of Cav2.1 at release sites (Kittel et al., 2006); it might therefore contribute to the additional recruitment of functional Cav2.1 during homeostatic presynaptic strengthening.

Using calcium imaging, a recent study by Muller and Davis (2012) revealed alteration of presynaptic Ca^{2+} influx upon induction of presynaptic homeostatic plasticity. They suggested that modulation of Ca^{2+} influx is sufficient to account for the rapid induction and maintenance of a homeostatic change in vesicle release. In agreement with this claim their analyses did not reveal variations in the number of docked vesicles released by hypertonic sucrose solution. Important to note, assaying the RRP by an alternative method (i.e., measurement of amplitudes of cumulative excitatory postsynaptic currents and fluctuation analysis) revealed changes in a comparable experimental design (Weyhersmuller et al., 2011; Muller et al., 2012). An imaging study employing a genetically coded Ca^{2+} sensor revealed that increased Ca^{2+} influx determines the induction of homeostatic changes of neurotransmitter release also in murine cultured hippocampal neurons (Zhao et al., 2011). However, whether the modulation of Ca^{2+} influx is achieved by increasing the number of Cavs or by modifying their gating properties requires further studies.

Other molecules required for homeostatic increase of presynaptic strength at NMJs are dysbindin and snapin, both involved in calcium-dependent release of SVs (Dickman and Davis, 2009; Dickman et al., 2012). These proteins seem to act downstream or independently of Cav2.1. *Dysbindin*, a gene linked to schizophrenia in humans, was identified in a screen for genes involved in homeostatic modulation of presynaptic release. It is localized to the SV cluster and might play a role in regulation of SVP (Dickman and Davis, 2009). Snapin interacts and acts together with dysbindin in synchronizing calcium-dependent SV exocytosis by interacting with the t-SNARE protein SNAP25 during membrane trafficking events (**Figure 1**; Dickman et al., 2012). Importantly to state, the studies on dysbindin/snapin also suggested a potential link between homeostatic signaling and neurological disease.

From the same screen for proteins playing a role in presynaptic homeostatic plasticity *gooseberry,* a pair-rule transcription factor that antagonizes Wingless (*Drosophila* homologue of Wnt) signaling during development, was isolated (Marie et al., 2010). Gooseberry is required for sustained expression of synaptic homeostasis and it possibly links Wingless signaling to this process. Recently, it was also shown that the expression of miR-310-313 microRNA cluster is required for normal synaptic homeostasis. miR-310-313 binds to the 3'UTR of the kinesin motor family member Khc-73 and attenuates its expression. When mutated, Khc-73 expression rises, which is accompanied by higher expression of bruchpilot and increased numbers of T-bars in presynapses (Tsurudome et al., 2010) These studies indicate that regulation of gene expression and new protein synthesis might contribute to homeostatic processes at presynapse.

Rab3 and Rab3-interacting molecule (RIM), both well-described regulators of synaptic vesicle release, were recently also shown to be necessary for presynaptic homeostasis in *Drosophila*. Rab3, a small GTPase present on SVs, when bound to GTP seems to suppress synaptic homeostastic adapation at a very late stage of synaptic vesicle exocytosis. At the same time, the Rab3 GTPase activating protein (Rab3-GAP), which regulates this inhibitory effect by accelerating GTP hydrolysis to convert Rab3-GTP into Rab3-GDP, turns out to be essential for expression of homeostatic plasticity (Muller et al., 2011). RIM is an evolutionary conserved scaffolding protein of the presynaptic AZ. Apart from being important in the maintenance of basal synaptic transmission, RIM is required for the homeostatic plasticity-evoked increase in the RRP of SVs (Muller et al., 2012). The modulation of the RRP by RIM

during homeostatic adaptation in *Drosophila* NMJ is a Ca^{2+}-independent process what implies that RIM is not involved in the inactivity-induced modulation of Ca^{2+} influx through Cav2.1 in this model system (Muller et al., 2012).

REGULATION OF SYNAPTIC VESICLE POOLS DURING HOMEOSTATIC PLASTICITY

In the pioneering work of Murthy et al. (2001) dealing with homeostatic plasticity in cultured hippocampal neurons it was demonstrated that changes supporting homeostatic scaling (inactivity-induced compensatory increase of quantal amplitude) were not only confined to postsynaptic terminals, as it was reported previously for the mammalian system (Turrigiano et al., 1998), but also affected the presynaptic machinery in a similar way as it was reported for the *Drosophila* NMJ. Imaging SV cycling via FM-dye loading showed that two days of activity blockade by interference either with action potential propagation using TTX or with AMPA receptor function by application of their antagonist NBQX led to an increase of the Pr and RRP size by about 50%. An electron microscopic analysis revealed an increase in the number of docked vesicles, the total number of vesicles in boutons and the area of AZs (**Figure 1**; Murthy et al., 2001). This study not only provided the first description of disuse-induced regulation of release in vertebrate neurons, but also supported the view that the RRP is formed by docked vesicles. A contrary approach, i.e., elevation of synaptic activity by depolarization, revealed a decrease in RRP (Moulder et al., 2006), and induced so-called synaptic muting, a phenomenon that will be discussed in detail below. Thus the RRP represents an important substrate for homeostatic plasticity and is bidirectionally regulated by overall activity levels to preserve stable firing rates. Multiple studies demonstrated that the total SVP and the TRP were also regulated during homeostatic adaptation to overall activity levels (Murthy et al., 2001; Thiagarajan et al., 2005; Branco et al., 2008; Lazarevic et al., 2011), nevertheless, the molecular players involved in this regulation remain poorly understood.

A key role in the regulation of the RtP during homeostatic plasticity was suggested recently for cyclin-dependent protein kinase 5 (CDK5; Kim and Ryan, 2010). The acute pharmacological inhibition of CDK5 activity resulted in an unmasking of previously silent synapses and in a mobilization of SVs of RtP resulting in the relative increase of the TRP. Neurons from CDK5 knock-out mice showed larger TRPs and in contrast to wild-type neurons, they did not further increase their TRP in response to inactivity. The regulation of CDK5 activity during inactivity occurs via regulation of protein turnover as inactivity led to a significant decrease in CDK5 expression levels. The protein phosphatase calcineurin B (CNB) seems to antagonize CDK5 in this regulation as neurons from CNB knock-out mice had a strongly reduced TRP. Thus the balance between CDK5 and CNB activity seems to determine the size of the RtP and the amount of vesicles available for action potential-driven release (**Figure 1A**). Nevertheless, the mechanism of CDK5 regulation during homeostatic plasticity remains to be addressed in future studies. Alterations of synaptic CDK5 amounts might be achieved by regulation at the level of gene expression, protein synthesis or degradation, which all reported to be involved in homeostatic plasticity-induced regulation of other protein targets.

Regulation of CDK5 activity by dynamic association with its activator p25/p35 was shown in a recent study investigating the effects of activity withdrawal in hippocampal organotypic slices (Mitra et al., 2012) further confirming a key role of this kinase during homeostatic synaptic plasticity. However, the possible targets of CDK5 in this process are not known. Synapsin, a SV-associated protein, is phosphorylated by CDK5, which controls its recruitment to synapses (Easley-Neal et al., 2013) and the mobility of vesicles belonging to the so called "superpool" (Orenbuch et al., 2012). This is a population of SVs capable of traveling along axons and taking part in transmission at different presynaptic terminals (Krueger et al., 2003; Darcy et al., 2006; Westphal et al., 2008; Staras et al., 2010). Although more studies need to be performed to understand the function of the "superpool," it seems likely that this mobile pool of vesicles can contribute to homeostatic adaptations (**Figure 1**), either by modulating SV pool sizes, mainly the RtP, or by acting as a readily accessible extrasynaptic pool (Staras and Branco, 2010).

Many further CDK5 substrates have been identified, which might potentially contribute to the modulation of presynaptic function during homeostatic adaptation; e.g., Munc18 (Fletcher et al., 1999), CASK (Samuels et al., 2007), or N-type Ca^{2+} channels (Su et al., 2012). However, the exact contribution of their modulation by CDK5 during homeostatic adaptations still needs to be elucidated.

The existence of the RtP and its contribution to the presynaptic plasticity was questioned in a recent study focusing on the CA3 to CA1 synapses in hippocampal slice cultures, where RtP could only be detected in immature (day *in vitro* 4) but not in mature (day *in vitro* 14–20) preparations (Rose et al., 2013). Nevertheless, strong depolarization in this system also led to the emergence of a release-incapable RtP. Similarly, pharmacological inhibition of CNB resulted in an increased RtP; this was, however, not statistically significant. Thus, it is possible that at mature CA1 to CA3 synapses the RtP emerges merely in reaction to pathophysiological situations such as stroke or seizures to reduce synaptic output and to prevent excitotoxicity.

HOMEOSTATIC ADAPTATION OF VESICULAR FILLING

Besides altering the mode of SV release, homeostatic synaptic plasticity also comprises changes in the vesicular filling with neurotransmitters. At hippocampal synapses, glutamate receptors are not saturated by quantal release (McAllister and Stevens, 2000). Thus, changes in the amount of glutamate released from a single vesicle modulate the strength of glutamatergic neurotransmission. Studies in *Drosophila* and mammals revealed that the number and type of the vesicular transporter proteins present in the vesic membrane determine the amount and type of neurotransmitter loaded into SVs (Wojcik et al., 2004, 2006; Wilson et al., 2005; Daniels et al., 2006).

De Gois et al. (2005) demonstrated that mRNA and protein expression of glutamate transporters VGLUT1 and VGLUT2 as well as of the GABA transporter VIAAT/VGAT are regulated by activity levels in cultured neocortical cells. Withdrawal of network activity by application of TTX for 2 days leads to an up-regulation of VGLUT1 mRNA and protein levels, whereas VGLUT2 and VIAAT/VGAT were significantly down-regulated. Treatment with

the $GABA_AR$ blocker bicuculline (Bic) for 2 days that increased overall network activity resulted in a decrease of VGLUT1, but in an enhancement of VGLUT2 and VIAAT/VGAT expression. These activity dependent changes in expression of transporters are reflected in a modulation of their synaptic abundance (De Gois et al., 2005; Lazarevic et al., 2011). It was demonstrated that over-expression of VGLUT1 in mammals or DVGLUT in *Drosophila* led to its increased incorporation into the SV membrane resulting in increased glutamate loading and release per vesicle (Daniels et al., 2004; Wilson et al., 2005). Therefore, activity-dependent modulation of transporter expression also leads to the differences in vesicular loading and consequently to modulation of quantal size (Wilson et al., 2005). In *Drosophila*, an over-expression of DVGLUT was compensated by a decrease in number of released vesicles (Daniels et al., 2004), which suggested the existence of a homeostatic mechanism that compensates for excessive excitation due to increase in glutamate release and is reminiscent to presynaptic muting described in mammals (see "Molecular mechanisms of presynaptic muting").

Vesicular glutamate transporter 1 and VGLUT2 being oppositely regulated by activity are expressed in distinct neuronal populations, which are only partially overlapping, and it is possible that differential regulation of both glutamate transporters plays a role in homeostatic shaping of circuit function. It was suggested that VGLUT2 is preferentially expressed at excitatory synapses contacting inhibitory neurons. Thus an activity-dependent regulation of VGLUT2 (Doyle et al., 2010) and VIAAT/VGAT, with opposite magnitude comparing to VGLUT1, might represent an intrinsic way for neurons to adjust their vesicular transmitter stores that are available for release to maintain or restore the E/I balance (De Gois et al., 2005). In agreement, a reduction of inhibitory transmission due to reduced SV filling by GABA was demonstrated upon chronic activity inactivation in cultured hippocampal neurons (Hartman et al., 2006).

Not only vesicular transporters but also enzymes involved in neurotransmitter synthesis might be regulated to contribute to presynaptic homeostatic adaptation. Lau and Murthy (2012) demonstrated recently that expression of glutamic acid decarboxylase 67 (GAD67), which is the rate-limiting enzyme in GABA synthesis, is regulated by global network activity and that this regulation affects the filling of SVs with neurotransmitter. Chronic suppression of activity with TTX resulted in decrease of GAD67 expression, reduced levels of GABA and lower mIPSC. Opposite changes in GAD67 expression and GABA levels were observed when network activity was elevated by prolonged mild depolarization of neurons or by treatment with $GABA_AR$ blocker picrotoxin. (Lau and Murthy, 2012)

Taken together, expression of vesicular transporters and enzymes involved in the synthesis of neurotransmitters regulates the levels of released neurotransmitters and therefore these proteins are critical determinants for the scaling of quantal size within physiological limits.

MODIFICATION OF RELEASE APPARATUS BY HOMEOSTATIC SYNAPTIC PLASTICITY

The alterations in RRP are the hallmark of virtually all forms of homeostatic adaptation at presynapse. Despite of their repetitive description, the underlying molecular mechanisms are not fully understood. One of the first described molecular changes correlating with an enhancement of presynaptic activity upon chronic blockade of glutamate receptors was the down-regulation of the interaction between synaptophysin and synaptobrevin/vesicle-associated membrane protein 2 (VAMP2; Bacci et al., 2001). Synaptophysin was suggested to bind synaptobrevin/VAMP2 and thereby hinder it to assemble into SNARE fusion complex required for exocytosis (Edelmann et al., 1995).

Several studies reported regulation of expression levels of synaptic proteins during presynaptic homeostatic adaptation to alterations in global network activity (Thiagarajan et al., 2005; Cohen et al., 2011; Lazarevic et al., 2011; Weyhersmuller et al., 2011). This suggested that functional presynaptic alterations might be connected with molecular remodeling of the release machinery. The main candidates for regulation were proteins functioning in neurotransmitter release such as SV proteins, Cavs and components of cytomatrix at the active zone (CAZ) implicated in the regulation of presynaptic release (Gundelfinger and Fejtova, 2012; Sudhof, 2012). We tested systematically changes in the expression of these proteins using quantitative immunoblotting and immunostainings at the level of single synapses (Lazarevic et al., 2011). In our experiments, we found a significant up-regulation of synaptic vesicle proteins, which is in good agreement with increased SVP upon network inactivation reported previously (Murthy et al., 2001). On the other hand, inactivity induced by prolonged (48-h) blockage of glutamate receptors resulted in the down-regulation of cellular expression levels of presynaptic scaffolding proteins bassoon, piccolo, ELKS/CAST, Munc13, RIM, liprin-α, and synapsin. This was accompanied by a general reduction of bassoon, piccolo, ELKS/CAST, Munc13 and synapsin levels at individual synaptic sites, whereas RIM was up-regulated in a subpopulation of synapses suggesting its redistribution upon activity withdrawal (**Figure 1B**). Interestingly, the amounts of RIM correlated well with activity levels, when analyzed at individual synapses, suggesting a role of RIM in defining the presynaptic probability of SV release in normally active and silenced cultures (Lazarevic et al., 2011). RIM is a multifunctional protein regulating the RRP size likely by binding to the priming factor Munc13, what in turn leads to release of Munc13 from an autoinhibitory complex and its activation (Deng et al., 2011). The key role of RIM in regulating the RRP during homeostatic plasticity was also confirmed by studies in *Drosophila* (Muller et al., 2012) and in studies on mechanisms of synaptic muting (Jiang et al., 2010), which will be discussed later.

Using combined imaging of a genetically encoded Ca^{2+} reporter localized on the synaptic vesicles and a reporter of synaptic vesicles fusion, Zhao et al. (2011) showed that a decrease in the network activity in cultured hippocampal neurons causes an increase in the amount of Ca^{2+} entry into presynaptic boutons and an increase in Pr. Furthermore, they found a third-power relation between homeostatic changes in presynaptic Ca^{2+} influx and Pr, proposing that even small changes in the number and/or function of presynaptic Ca^{2+} channels might have large effects on synaptic strengths (Zhao et al., 2011). Accordingly, our data revealed an accumulation of the pore-forming subunit Cav2.1 of P/Q-type Cavs at the synapses of activity-deprived neurons (Lazarevic et al.,

2011). Recently, a novel role for RIM proteins in the localization of Cavs to the AZ was described (Han et al., 2011; Kaeser et al., 2011) and it is well possible that the homeostatic plasticity-induced changes in Ca^{2+} influx are dependent on RIM-mediated recruiting of Cavs to release sites. Moreover, the inactivity also induces enrichment of the Ca^{2+} sensor protein synaptotagmin1 (Lazarevic et al., 2011), which was also described to interact with RIM (Coppola et al., 2001; Schoch et al., 2002). Thus, RIM might contribute to the manifestation of homeostatic adaptations not only by controlling the vesicle priming but also by recruiting multiple interaction partners involved in coupling of release sites to Ca^{2+} signaling.

The extensive molecular remodeling of the release machinery induced by activity withdrawal requires regulation of protein turnover at synapses, which might be in principal driven by two mechanisms: (1) alteration of protein synthesis rates at transcriptional or translational level or (2) regulation of the selective removal of synaptic proteins, mostly via the ubiquitin-proteasome system (UPS; **Figures 1B,C**).

Synaptic vesicle glycoprotein 2A, a protein associated with SV and involved in priming process (Chang and Sudhof, 2009), has been recently shown to be a target of regulation by the microRNA miR-485 (Cohen et al., 2011). The expression levels of SV2A are reduced after chronic elevation of synaptic activity. Interestingly, inhibition of miR-485 function interferes with SV2A down-regulation indicating that the control of stability of certain mRNAs by microRNAs contributes to homeostatic adaptation. Although there are no concrete targets known yet, the regulation of gene expression at the level of transcription likely plays a role in the presynaptic homeostatic plasticity as inhibition of transcription interferes with increase in mEPSC frequency upon network activity silencing (Han and Stevens, 2009).

The UPS is emerging as a powerful modulator of synaptic function, acting at both postsynaptic and presynaptic sites (Hegde, 2010). Moreover, UPS was shown to regulate the turnover of presynaptic CAZ proteins RIM (Yao et al., 2007), Munc13 (Speese et al., 2003) and liprin-alpha (van Roessel et al., 2004). Recently, bassoon and piccolo were identified to control levels of presynaptic ubiquitination, which is at least partially mediated by their interaction with E3 ubiquitin ligase SIAH-1 leading to enzyme inhibition. Consequently, loss of these two major CAZ components promotes excessive ubiquitination and degradation of many presynaptic proteins, which results in synapse degeneration (Waites et al., 2013). We tested involvement of UPS in inactivity-induced down-regulation of CAZ proteins and demonstrated that UPS-driven degradation is highly substrate specific and that it is controlled by network activity. In our study, UPS-dependent degradation of bassoon and liprin-alpha was enhanced upon activity deprivation, but interestingly, RIM and Munc13 were unchanged (Lazarevic et al., 2011). These results suggest that alternative cellular mechanisms control the redistribution of RIM in response to activity withdrawal. However, as it will be discussed later RIM seems to be a target of UPS-mediated degradation during presynaptic muting (Jiang et al., 2010).

Taken together, intense molecular reorganization of release sites underlies the functional alteration of release during homeostatic presynaptic adaptation. Although, several effector proteins regulated during this process have been identified, the underlying signaling pathways and exact nature of their modification needs to be addressed by future studies.

MOLECULAR MECHANISMS OF PRESYNAPTIC MUTING

Adaptive presynaptic silencing or presynaptic muting is a form of presynaptic homeostatic adaptation preventing the runaway excitation or excitotoxicity during physiological and pathophysiological depolarization such as seizures or hypoxic insults. Presynaptic muting can be induced by rearing cultured dissociated hippocampal neurons or cerebellar granule neurons at elevated K^+ concentrations. It is manifested by selective functional inactivation of excitatory presynapses, which appear normal if assessed morphologically (Moulder et al., 2004, 2006). Presynaptic muting is due to a decrease in size of the RRP at individual AZs and does not require glutamate receptor activation or rises in intracellular Ca^{2+} from neither extracellular nor intracellular stores (Moulder et al., 2006; Crawford et al., 2011). The reduction of RRP is likely due to a block in the priming process as treatment with alpha-latrotoxin induces neurotransmitter release in muted synapses (Moulder et al., 2006). In parallel to the effect on RRP, prolonged depolarization induces depressed Pr, which is common to both glutamate and GABA transmission and likely due to reduced Ca^{2+} influx into presynaptic terminals (Moulder et al., 2003, 2004). The effects on Pr and RRP seem to be independent as their manifestation is separated temporally (with changes in the RRP seen already 4 h, but Pr only 16 h after depolarization) and segregated between glutamatergic and GABAergic synapses, whereas only the glutamatergic synapses express the muting (Moulder et al., 2004). Under physiological conditions a certain fraction of synapses remains unresponsive (silent) to action potentials and this fraction can be modulated in response to changes in physiological activity. This suggests that presynaptic muting also takes place under physiological conditions and might represent a mechanism, by which neuronal networks tune the excitatory drive depending on the levels of network activity (Moulder et al., 2006). Blockade of action potential propagation could reverse depolarization-induced muting, whereas block of glutamate receptors could not, what suggests a presynapse autonomous mechanism (Moulder et al., 2006).

Only a subset of synapses in depolarized cultures exhibit presynaptic muting and it is unknown, what are the molecular determinants of this variability. Presynaptic muting is reminiscent of the synaptic phenotype of mouse mutants for the CAZ proteins Munc-13 (Augustin et al., 1999; Rosenmund et al., 2002) and bassoon (Altrock et al., 2003), which display a reduction in numbers of functional synapses and deficits in SV priming. This suggests an involvement of CAZ proteins in the process of presynaptic muting. This assumption is supported by the finding that levels of CAZ proteins RIM and Munc-13 are decreased in muted synapses as compared to active ones, both under basal conditions and after depolarization induced synaptic muting (Jiang et al., 2010). The reduction of RIM expression levels at synapse are due to its degradation by the UPS; inhibition of UPS by the proteasomal blocker MG-132 fully inhibited the depolarization-induced decrease in RIM and also prevented presynaptic muting (**Figure 1C**). In line with this notion, over-expression of RIM1

completely prevented depolarization-induced synaptic muting as well as decrease of Munc-13 levels (Jiang et al., 2010) supporting previous assumptions on a function of RIM in maintaining the Munc13 synaptic levels (Schoch et al., 2002). Taken together, these experiments demonstrate that the modulation of RIM1 expression levels at synapse play a key role in the induction of presynaptic muting and also suggest an important role of the UPS in this process.

Cyclic-AMP (cAMP) signaling was also proposed to play a role in presynaptic muting as a treatment with forskolin, an activator of adenylate cyclases (AC) inducing an increase of cAMP levels, prevented induction of synaptic muting upon depolarization and reduced the fraction of muted synapses under basal conditions (Moulder et al., 2008). This pathway seems to interfere with the UPS in a more complex manner; forskolin treatment did not affect proteasomal activity but did affect synaptic levels of RIM1 and Munc13 (Jiang et al., 2010). To identify the molecular components of the cAMP signaling pathway involved in presynaptic muting, a requirement of the two main Ca^{2+}-sensitive ACs (types 1 and 8) was tested. The activity these two ACs is not needed to induce muting, but recovery from depolarization induced muting was strongly affected in neurons derived from knockout mice for AC8. The treatment with a broadly acting inhibitor of cAMP-dependent kinases or with specific PKA inhibitor, however, completely prevented recovery from presynaptic muting suggesting a contribution of multiple forskolin-sensitive ACs to control this process (**Figure 1C**; Moulder et al., 2008). In a follow-up study on this topic, presynaptic muting was completely prevented by pharmacological blockade of inhibitory G ($G_{i/o}$) proteins. The exact target of this treatment was not identified, but it was shown that activation of adenosin A1 and $GABA_B$receptors induced presynaptic muting, which was dependent on normal activity of the UPS. However, blockade of these receptors did not interfere with the induction of presynaptic muting by depolarization (Crawford et al., 2011).

Recovery from presynaptic muting can be induced by shifting neurons to media with physiological K^+ concentration, which leads to the establishment of normal membrane potential. Such "unmuting" is detectable after 3 h recovery, requires PKA activity (Moulder et al., 2008) and is blocked in the presence of transcription and translation inhibitors suggesting a role of protein synthesis in this process (Crawford et al., 2012b). Potential protein candidates synthesized during unmuting are RIM and Munc13. Their levels are reduced in muted synapses, recover during unmuting, but remain reduced if translation and transcription are blocked (Crawford et al., 2012b). It is not yet fully understood how PKA activation translates into regulation of protein synthesis. PKA regulates activity of CREB during unmuting (Crawford et al., 2012b), but it is likely that other transcription factors are also involved. Fast synaptic unmuting can also be induced by treatment with phorbol esters and is measurable within several minutes (Moulder et al., 2008; Chang et al., 2010). It is not dependent on proteins synthesis and does not require PKC activity. Likely it depends on direct interaction of phorbol esters with Munc13-1, one of the main priming/docking factors (Betz et al., 1998; Chang et al., 2010). Thus studies on presynaptic muting and unmuting propose a role of cAMP signaling in these processes, which results in tuning of turnover of key regulators of Pr, i.e., RIM and Munc13, and involves modulation of protein synthesis as well as protein degradation by UPS.

Recently, it was demonstrated that astrocyte deprivation prevented presynaptic muting. The role of thrombospondins, glycoproteins secreted by astrocytes, was proposed. Gabapentin, a high affinity antagonist of thrombospondins binding to its receptor α2δ, mimicked effect of astrocyte deprivation (Crawford et al., 2012a). α2δ functions also as an auxiliary subunit of Cavs and regulates their synaptic localization and function (Dolphin, 2012). Astrocyte deprivation leads to abnormal activity of PKA and increased phosphorylation of its target proteins such as synapsin, cAMP response element-binding protein (CREB). However, the link between α2δ-1 and PKA signaling remains unclear (Crawford et al., 2012a). This study put forward a novel mechanism, by which neuron - glia interaction might control synaptic homeostasis.

PRESYNAPTIC HOMEOSTATIC PLASTICITY AND BRAIN DISEASE

Numerous brain dysfunctions are connected with disturbed physiological synaptic functions leading to changes in the activity of brain circuits, which in turn induces maladaptive plasticity. The mechanisms of induction and the consequences of such maladaptive plasticity closely relates to homeostatic plasticity.

Dickman and Davis (2009) demonstrated a critical role of *dysbindin*, a gene linked to schizophrenia in humans, in homeostatic presynaptic adaptations in *Drosophila* NMJ. In vertebrates, dysbindin is associated with SVs (Talbot et al., 2006) and is involved in the regulation of glutamate release (Numakawa et al., 2004). In schizophrenia patients, dysregulation of glutamatergic transmission and reduced expression of dysbindin were reported (Mechri et al., 2001; Talbot et al., 2004, 2006; Weickert et al., 2004, 2008; Tang et al., 2009), suggesting that dysfunction of dysbindin may result in failure of homeostatic plasticity mechanisms tuning glutamatergic transmission.

Presynaptic muting was proposed to play a key role in protecting neurons from damage due to glutamate excitotoxicity occurring during epileptic seizures, hypoxia or ischemia (Hogins et al., 2011). The preconditioning by mild depolarization, which induces synaptic muting by UPS-dependent mechanisms (Moulder et al., 2004; Jiang et al., 2010), protected neurons from damage induced by hypoxia or oxygen/glucose deprivation. Blockade of UPS during preconditioning completely abolished the preconditioning induced protection. Hypoxia itself induced muting in a proteasome-dependent manner; UPS inhibition exacerbated neuronal loss upon mild hypoxia and prevented hypoxia-induced muting. These data suggest that overexcitation-induced presynaptic muting provides endogenous neuroprotection mechanisms to limit the damage from insults involving excess synaptic glutamate release (Hogins et al., 2011).

Several presynaptic proteins involved in the homeostatic regulation of neurotransmitter release were linked to epilepsy, where excessive synaptic activity occurs leading to glutamate-induced excitotoxicity. Mice mutant for the SV protein SV2A exhibit spontaneous epileptic seizures (Crowder et al., 1999; Janz et al., 1999) similarly as do mice mutant for synapsin (Rosahl et al., 1995) or

the AZ protein bassoon (Altrock et al., 2003) suggesting important roles of these proteins in the control of baseline neuronal activity at the organismal level. Interestingly, a recent study has shown that mice mutant for protein RIM1aplha show a dramatically increased frequency of spontaneous recurrent seizures upon experimental induction of epilepsy, despite of the fact that these mice do not show any changes in the basal activity patterns assessed by EEG (Pitsch et al., 2012). This phenotype is consistent with the proposed key role of RIM regulation in the induction of presynaptic muting (Jiang et al., 2010) and with the idea that presynaptic homeostatic plasticity mechanisms such as presynaptic muting are recruited for dampening synchronous activity-induced epileptogenesis.

Homeostatic synaptic plasticity, which constantly acts to stabilize neuronal networks whenever Hebbian plasticity or pathological synapse dysfunction alter the synapse weight, turned out to be a key mechanism ensuring the long-term functioning of brain circuits. Accordingly, cellular mechanisms controlling homeostatic synaptic plasticity, as well as underlying molecules and signaling pathways represent emerging targets for drug development and new therapeutic strategies for various neurological and psychiatric disease conditions.

ACKNOWLEDGMENTS

We thank E. D. Gundelfinger for critical reading of this article. Anna Fejtova is supported by DFG FE 1335/1 and by the federal state of Saxony-Anhalt, Project: Center for Behavioral Brain Sciences (CBBS). The research leading to these results has received funding from the People Programme (Marie Curie Actions) of the European Union's Seventh Framework Programme FP7/2007-2013/ under REA grant agreement no [289581].

AUTHOR CONTRIBUTIONS

Vesna Lazarevic, Santosh Pothula, Maria Andres-Alonso and Anna Fejtova wrote the paper, Vesna Lazarevic prepared the figure. Vesna Lazarevic, Santosh Pothula and Maria Andres-Alonso contributed equally to this work

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 11 September 2013; paper pending published: 29 September 2013; accepted: 18 November 2013; published online: 03 December 2013.

*Citation: Lazarevic V, Pothula S, Andres-Alonso M and Fejtova A (2013) Molecular mechanisms driving homeostatic plasticity of neurotransmitter release. Front. Cell. Neurosci. **7**:244. doi: 10.3389/fncel.2013.00244*

This article was submitted to the journal Frontiers in Cellular Neuroscience.

frontiers in
CELLULAR NEUROSCIENCE

REVIEW ARTICLE
published: 20 January 2015
doi: 10.3389/fncel.2014.00439

Structural homeostasis in the nervous system: a balancing act for wiring plasticity and stability

Jun Yin and Quan Yuan*

National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD, USA

Edited by:
Jaichandar Subramanian, Massachusetts Institute of Technology, USA

Reviewed by:
Gaia Tavosanis, German Center for Neurodegenerative Diseases, Germany
Matthias Landgraf, University of Cambridge, UK

***Correspondence:**
Quan Yuan, National Institute of Neurological Disorders and Stroke, National Institutes of Health, 35 Convent Drive, 1B-1002, Bethesda, MD 20892, USA
e-mail: quan.yuan@nih.gov

Experience-dependent modifications of neural circuits provide the cellular basis for functional adaptation and learning, while presenting significant challenges to the stability of neural networks. The nervous system copes with these perturbations through a variety of compensatory mechanisms with distinct spatial and temporal profiles. Mounting evidence suggests that structural plasticity, through modifications of the number and structure of synapses, or changes in local and long-range connectivity, might contribute to the stabilization of network activity and serve as an important component of the homeostatic regulation of the nervous system. Conceptually similar to the homeostatic regulation of synaptic strength and efficacy, homeostatic structural plasticity has a profound and lasting impact on the intrinsic excitability of the neuron and circuit properties, yet remains largely unexplored. In this review, we examine recent reports describing structural modifications associated with functional compensation in both developing and adult nervous systems, and discuss the potential role for structural homeostasis in maintaining network stability and its implications in physiological and pathological conditions of the nervous systems.

Keywords: structural plasticity, homeostasis, neural development, neuronal morphology, activity-dependent plasticity

INTRODUCTION

The structural organization of the nervous system has been studied since the earliest days of neuroscience. Before the wide use of electrophysiology, anatomical study was the main approach for neuroscientists to investigate the organization of the nervous system and infer principles governing its operation. Since then, samples from various species and developmental stages revealed remarkable complexity, diversity, and flexibility in neuronal forms and connections, which are mainly determined by each individual's genetic composition, but also largely influenced by experience and environmental factors (Holtmaat and Svoboda, 2009; Fu and Zuo, 2011). Although most prominent during development, structural plasticity is also evident in adult brains, serving critical cognitive functions such as learning and memory (Goodman and Shatz, 1993; Katz and Shatz, 1996; Chklovskii et al., 2004; Lamprecht and LeDoux, 2004).

As a fundamental property of the nervous system, its functional and structural flexibility provides the ability to adapt and incorporate genetic, developmental, and environmental variations, but at the same time, poses significant challenges to the integrity of neural networks. Therefore, counterbalancing mechanisms that maintain network stability are critically important. Observations in the central and peripheral nervous systems of various model organisms validated the existence of compensatory regulatory mechanisms, which are defined as neuronal homeostasis (Turrigiano and Nelson, 2000; Davis, 2013). In contrast to the classic Hebbian form of plasticity, where positive feedback regulation reinforces activity-induced changes and leads to long-lasting synaptic plasticity (Turrigiano and Nelson, 2000; Malenka and Bear, 2004; Cooper and Bear, 2012), homeostatic plasticity constrains network activity within the target physiological limit in response to changes of synaptic or intrinsic activity (Davis and Bezprozvanny, 2001; Turrigiano and Nelson, 2004; Marder and Goaillard, 2006; Turrigiano, 2012). Hebbian and homeostatic plasticity are opposing forces that potentially drive neuronal changes in different directions. Recent findings revealed both convergent and distinct molecular pathways underlying these two forms of plasticity. Mechanisms regulating their intricate interplay are clearly important for the nervous system to achieve proper balance between flexibility and stability, but remain largely unknown (Vitureira and Goda, 2013).

Based on the current understanding of underlying cellular mechanisms, neuronal homeostasis is generally categorized as the homeostatic control of: (a) intrinsic excitability through the regulation of ion channel expression; (b) synaptic efficacy through the synaptic scaling at the postsynaptic density (PSD); (c) release of presynaptic neurotransmitter; and (d) network activity through regulation of inhibitory synapses (Turrigiano and Nelson, 2000, 2004; Davis, 2013). Most of the studies related to neuronal homeostasis focused on modulation occurring at the level of synaptic physiology. However, under conditions where synaptic homeostasis is induced, concomitant morphological changes were also observed (Murthy et al., 2001; Butz et al., 2009b; Keck et al., 2013). More importantly, it is clear that some of the activity-dependent

structural plasticity did not follow the Hebbian learning rules, since they act on a global scale rather than being restricted within specific synapses, and produce counterbalancing rather than reinforcing effects on activity-driven changes, suggesting a structural realization of the homeostatic regulation (Tripodi et al., 2008; Butz et al., 2009b; Yuan et al., 2011; Keck et al., 2013). Are these structural modifications merely adaptive responses reacting to the alterations in synaptic physiology, or a part of the homeostatic mechanism that is actively contributing to the stabilization of the network activity? Answering this question is potentially important for us to gain a comprehensive understanding on how neural plasticity is cooperatively regulated through distinct mechanisms, and how the balance between wiring dynamics and network stability are achieved and modified during development and in adulthood. Here, we summarize observations generated from a diverse group of model systems that suggest a close association between structural plasticity and neuronal homeostasis, and discuss how these studies might support structural homeostasis as a conserved mechanism in regulating neuronal function, as well as its implications in physiological and pathological conditions.

STRUCTURAL PLASTICITY ASSOCIATED WITH HOMEOSTASIS AT THE EXCITATORY SYNAPSE

Activity-dependent structural plasticity at the excitatory synapse has been an intensely investigated research topic for decades. Structural modifications accompanying experience, such as spine growth induced by learning, are proposed to be the cellular mechanism underlying cognitive functions and behavioral plasticity (Chklovskii et al., 2004; Lamprecht and LeDoux, 2004; Holtmaat and Svoboda, 2009). In mammalian nervous systems, the organization and composition of excitatory synapses are well characterized. They generally form between the presynaptic bouton, containing the presynaptic active zone (AZ) with the readily releasable neurotransmitter vesicles, and the postsynaptic spine, containing the PSD that organizes neurotransmitter receptor clusters. In general, the size of a synapse or synaptic components is positively correlated with the synaptic efficacy and strength. Neuronal activity can strongly influence the size and structure of a synapse, as well as its distribution and dynamics (**Figure 1**; Murthy et al., 2001; Holtmaat and Svoboda, 2009).

Many classic studies on neuronal homeostasis focused on alterations in synaptic strength and efficacy upon chronic changes in neuronal activity, either through pharmacological treatments that artificially increase or decrease neural activity, or sensory deprivation (Turrigiano and Nelson, 2000; Turrigiano, 2008; Pozo and Goda, 2010; Cooper and Bear, 2012), while structural modifications were also observed under similar induction protocols. For example, early morphological studies in hippocampal slices from adult rat showed that dendrites became more spiny in slices with blocked synaptic transmission (Kirov and Harris, 1999). In a series of experiments that established homeostasis as a distinct form of synaptic plasticity, chronic blockade of cortical culture activity with the sodium channel blocker tetrodotoxin (TTX) not only enhanced synaptic strength through synaptic scaling at the PSD (Turrigiano et al., 1998), but also led to increased synaptic size, with all synaptic components, including the AZ, PSD and the bouton becoming larger (Murthy et al., 2001). In addition, to improve spatial and temporal resolutions to manipulate neuronal activity, researchers used ectopic expression of potassium channel Kir2.1 to suppress excitability in cultured hippocampal neurons (Burrone et al., 2002). Chronic suppression of activity after synapse formation in individual neurons within an active network led to homeostatic increase in synaptic input strength, and the total recycling pool of vesicles enlarged in synapses terminating on Kir2.1-expressing neurons (Burrone et al., 2002). Importantly, this study also indicated that timing of the activity modification and competitions among synapses strongly influence expression of synaptic homeostasis.

Taking advantage of the advancement in imaging techniques, *in vivo* experiments in mammalian sensory systems provided additional evidence for the structural changes associated with neuronal homeostasis. In the somatosensory system, long-term sensory deprivation in mice through whisker trimming results in a reduced rate of ongoing spine elimination in the barrel cortex. Since there are continuous synapse and spine loss after

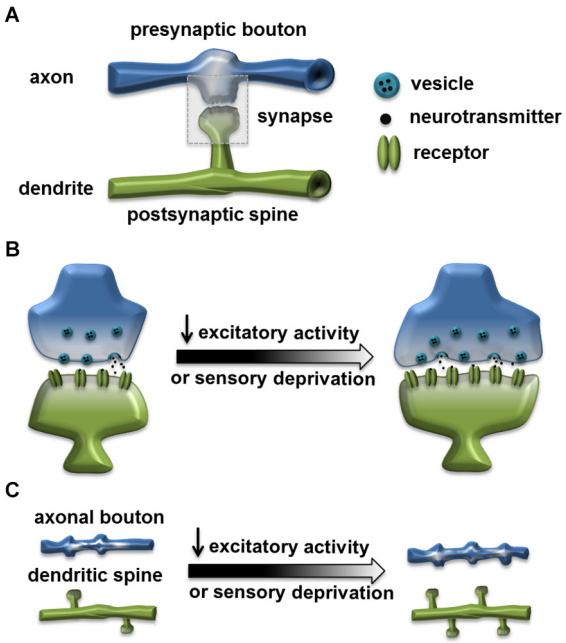

FIGURE 1 | Homeostatic structural change at the excitatory synapse. **(A)** A typical excitatory synapse. Synapse is formed through connection of the presynaptic axonal bouton, containing the presynaptic active zone (AZ) with the readily releasable neurotransmitter vesicles, and the postsynaptic dendritic spine, containing the postsynaptic density (PSD) that organizes neurotransmitter receptor clusters. **(B)** Reduced excitatory activity or sensory deprivation can lead to structural changes including: enlarged spine, increased size of active zone and increased number of vesicles (Murthy et al., 2001; Wallace and Bear, 2004; Keck et al., 2013). **(C)** Reduced excitatory activity or sensory deprivation can also lead to an increased axonal bouton number (Yamahachi et al., 2009; Kremer et al., 2010); increased dendritic spine number (Kirov and Harris, 1999); or reduced spine elimination rate (Zuo et al., 2005).

birth, the reduced spine elimination might be a way to compensate for the loss of sensory inputs (Zuo et al., 2005). More recently, studies were carried out in the mouse visual cortex with sensory deprivation generated through precise focal retinal lesions. The synaptic activity in the cortical neurons was initially decreased significantly, but gradually recovered within 2 days. This was coincident with enlarged spine size in layer 5 pyramidal neurons in the projection zone (Keck et al., 2013).

As an important mechanism for neuronal homeostasis, homeostatic regulation of presynaptic neurotransmitter release was demonstrated in the *Drosophila* neuromuscular junction (NMJ; Davis and Bezprozvanny, 2001; Frank, 2014). Depleting postsynaptic glutamate receptor subunits GluRIIA or GluRIIC reduced quantal size and elevated presynaptic release (Petersen et al., 1997; DiAntonio et al., 1999; Marrus et al., 2004). Moreover, a number of genetic mutations associated with diminished glutamate receptor clusters at NMJ also show reduced quantal size coupled with a compensatory increase in quantal content (Albin and Davis, 2004; Heckscher et al., 2007). Although the homeostatic regulation of synaptic physiology is very robust in NMJ, clear compensatory structural modifications were not observed in this system, suggesting that expression of synaptic homeostasis may be strongly influenced by induction methods and neuronal types.

Taken together, there is evidence suggesting that structural modifications of synaptic compartments are associated with the neuronal homeostasis. However, the results are mixed and it is clear that functional synaptic homeostasis can exist alone without obvious morphological alterations. Current limited data suggest that structural homeostasis may share molecular mechanisms with functional synaptic homeostasis. In several cases, structural homeostasis is correlated with "synaptic scaling" which involves trafficking of postsynaptic AMPA and NMDA receptors. In visually deprived animals, the number of AMPA receptors in the spine increased in parallel with the enlarged neuron spine head (Wallace and Bear, 2004; Keck et al., 2013). Mice with blocked NMDA receptors showed decreased spine elimination rate in the brain, similar to the homeostatic change in dynamics caused by whisker trimming (Zuo et al., 2005). But, in general, the information on molecular signaling pathways for structural synaptic homeostasis remains largely unknown. It is likely that physiological and structural changes collaborate in the process of re-establishing network stability, but occur within different temporal and spatial scales, and might be induced through both shared and distinct mechanisms.

LARGE-SCALE STRUCTURAL MODIFICATIONS ASSOCIATED WITH NEURONAL HOMEOSTASIS

Although some homeostatic regulations act in a synapse-specific manner, in general, neuronal homeostasis involves organized responses within a neuron or a neural network composed of many synaptic connections (Turrigiano, 2012). Therefore, to better understand the relation between structural and functional homeostatic plasticity, it is essential to analyze the overall change of network structure with correlative physiology data. This is challenging because of the extensive neuronal projections and synaptic connections made by mammalian cortical neurons. Nonetheless, several groups demonstrated large-scale reorganizations of axonal or dendritic compartments associated with homeostasis in both vertebrate and invertebrate systems.

AXONAL SPROUTING ASSOCIATED WITH REDUCED VISUAL ACTIVITY

Activity-dependent reorganization at the level of axonal and dendritic arbors is traditionally associated with the developing nervous system (Antonini and Stryker, 1993; Portera-Cailliau et al., 2005). In an adult brain, neuronal connections remain relatively stable and most of the structural modifications are observed at the synaptic level. Yet, large scale structural changes, such as axonal or dendritic arbor dynamics were demonstrated in adult brains, providing the cellular basis for the sustained structural flexibility in fully developed neural circuits (Fu and Zuo, 2011). For example, De Paola et al. (2006) reported prominent plasticity of axonal arbors in mouse barrel cortex through long-term *in vivo* imaging and demonstrated important rewiring ability in the axonal compartment of mature neurons.

Specifically, *in vivo* imaging studies in the primate visual cortex showed compensatory axonal sprouting associated with reduced visual activity. In animals with retinal lesions, an increased number and turnover rate of axonal boutons in the lesion projection zone (LPZ) were observed (Yamahachi et al., 2009). Moreover, focal binocular retinal lesions generated large-scale axonal sprouting and pruning in long-range horizontal axons within the LPZ, followed by proliferation of the horizontal axons at a high rate with density peaking within 1 week. Although the axon elimination rate also increased in the later stage, the overall axon density remained elevated during the whole observation period of 7–8 weeks, suggesting a large-scale and long-lasting reorganization of axonal projections in response to reduced visual input (Yamahachi et al., 2009).

HOMEOSTATIC STRUCTURAL TUNING OF THE AXON-INITIAL SEGMENT

The axon-initial-segment (AIS) is a highly specialized structure that separates axonal and somato-dendritic compartments. Axon-initial-segment maintains neuronal polarity by filtering the cellular cargo, functions as the trigger zone for action potentials, and is involved in modulating complex neuronal processing (Grubb et al., 2011). Shifting the AIS location and changing its size potentially affect the speed of electric signal propagation and the intrinsic excitability of the neuron. With its unique molecular composition, AIS provides an opportunity for morphological and functional studies following activity modification (Grubb and Burrone, 2010; Gründemann and Häusser, 2010; Kuba et al., 2010; Kuba, 2012).

Two research groups examined activity-dependent structural dynamics of the AIS recently and convincingly demonstrated homeostatic structural tuning in vertebrate neurons. In cultured hippocampal neurons, chronically elevated intrinsic activity through the action of channel rhodopsin (ChR2), or increased extracellular potassium, resulted in a distal shift of AIS from the soma and a reduction in AIS length (Grubb and Burrone,

2010). Along with this distal shift, there was a relocation of AIS-specific proteins including several ion channel components. This distal movement of ion channels reduced the ability of the input to trigger action potentials. Thus, the movement of the AIS away from the soma reduced excitability to compensate for increased neuronal activity. Notably, the AIS relocation through elevated activity was reversible, as AIS shifted proximally toward the soma after the activity returned to baseline, suggesting a regulatory mechanism for fine-tuning the AIS structure base on the ongoing activity (**Figure 2A**; Grubb and Burrone, 2010).

Similar observations were made in neurons in the nucleus magnocellularis (NM) of the chick auditory system, where sound frequency tuned the structural properties of AIS bi-directionally. High-frequency sounds produced less auditory input and led to shorter AIS, and vice versa (Kuba and Ohmori, 2009). In a follow-up study, Kuba et al. (2010) further showed that, when auditory activity was abolished by removing the cochlea in chickens, the AIS in deprived NM neurons elongated more than 50% and its distance to the soma decreased as well.

Using complementary approaches, these two studies clearly demonstrated homeostatic modification of the length and location of AIS. These observations strongly suggest that homeostatic structural plasticity of AIS can effectively modulate neuronal function and serve as a mechanism for tuning neuronal activity based on sensory input.

HOMEOSTATIC MODIFICATION OF THE DENDRITIC ARBORS IN *DROSOPHILA* CENTRAL NEURONS

Drosophila neurobiology has contributed significantly to our knowledge of the principles governing neural development and circuit organization. Recent studies demonstrated structural modifications associated with neuronal homeostasis in several types of *Drosophila* central neurons. The first example of homeostatic remodeling of dendritic arbors came from the study on aCC, a group of embryonic motor neurons located in the ventral nerve cord and receiving cholinergic inputs (Tripodi et al., 2008). Blocking the neurotransmitter synthesis or evoked release from presynaptic cholinergic neurons both led to expansion of the aCC dendritic arbor. Conversely, when the density of presynaptic contacts and synapses formed on dendrites was increased by genetic manipulation, the size of the aCC dendritic arbor was significantly reduced (**Figure 2B**). The authors proposed that the dendrite of aCC motor neurons exhibits activity-dependent structural homeostasis, which could serve as a compensatory mechanism for neurons to cope with the variation of presynaptic inputs throughout development (Tripodi et al., 2008).

The mushroom body is a well-studied structure in the *Drosophila* central nervous system (CNS) due to its close association with learning and memory, sensory integration and behavioral plasticity. Early studies showed that the volume of mushroom body can be modified by culture conditions and sensory experience (Heisenberg et al., 1995). Recently, Kremer et al. (2010) studied experience-dependent modification of synaptic structures in the adult mushroom body through high-resolution imaging. What they have found was unexpected for the structure associated with memory, where lack of input was assumed to lead to reduced complexity and volume. Instead, when input activity was silenced through ectopic expression of a potassium channel dORK1.ΔC in presynaptic projection neurons, they observed a significant increase in the density and size of the microglomerulus, the synaptic complex formed between axon terminals from projection neurons and postsynaptic structure of Kenyon cells in the mushroom body. This suggests a possible homeostatic upregulation of the microglomerulus synapse in response to suppression of neuronal activity (Kremer et al., 2010). However, the activity manipulation was carried out only in presynaptic neurons and it was not restricted to adulthood. With improved temporal control and additional studies in postsynaptic mushroom body neurons, future work using this system might provide us with more information regarding the extent and location of these homeostatic structural changes, as well as how this type of regulation interacts with the sensory integration and learning activity in the mushroom body.

Our study using the developing larval visual system further demonstrated structural homeostasis in *Drosophila* CNS and depicted novel features and molecules involved in its regulation (Yuan et al., 2011). In *Drosophila* larvae, presynaptic photoreceptors send an axonal projection to the brain and make synaptic contacts with dendritic arbors of ventral lateral neurons (LNv). Light stimulation-induced synaptic activity produced striking changes in the length of LNv dendrites, with the amount of

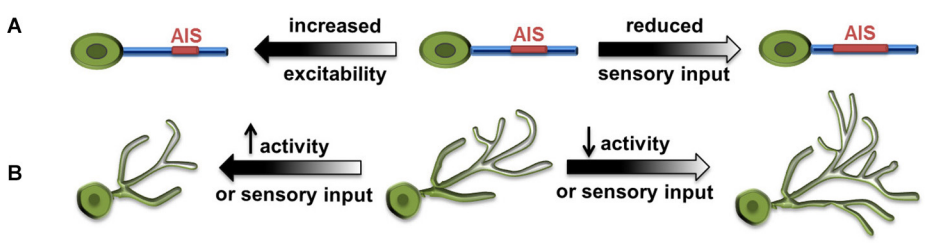

FIGURE 2 | Large-scale homeostatic structural changes. (A) Increased excitability results in distal shift of axonal initial segment (AIS) from the soma and reduced AIS length (Grubb and Burrone, 2010), whereas reduced sensory input results in increased AIS length and reduced distance to soma (Kuba et al., 2010). **(B)** Increased synaptic activity and excitability, as well as increased sensory input lead to decreased size of dendritic arbors. Conversely, reduced activity or visual input leads to increased size of dendritic arbors (Tripodi et al., 2008; Yuan et al., 2011).

light exposure received by the animal inversely correlated with the total dendritic length of LNv (Yuan et al., 2011). Through neuronal-specific alterations of activity and neurotransmission, we demonstrated compensatory structural changes in dendritic arbors driven by either sensory experience, or alterations in the presynaptic neurotransmission or synaptic activity, or changes in the intrinsic excitability in postsynaptic neurons (**Figure 2B**). This large-scale, bi-directional and homeostatic structural plasticity is accompanied by changes in the LNvs' physiological output. Light-evoked activity in LNvs, measured by calcium imaging, are also modified by experience, where increased light exposure correlated to reduced dendrite length and lower physiological response in LNvs. This observation clearly contrasts with the classic homeostasis theory, in which a set point that precisely defines the network output is a major feature (Davis, 2006).

In combination, these three studies demonstrated structural homeostasis associated with distinct developmental stages: embryonic motor neurons undergoing active synaptogenesis, larval LNv extending its connectivity with the expansion of the brain volume, and adult mushroom body neurons responding to the learning or experienced-dependent modification. These results support the idea that *Drosophila* central neurons can continually utilize structural modifications as a mechanism for modifying functional output according to developmental and environmental influences.

STRUCTURAL PLASTICITY ASSOCIATED WITH NEURONAL HOMEOSTASIS AT THE INHIBITORY SYNAPSE

Many previous studies focused on modification of excitatory synapses formed on spines, partly due to their accessibility in morphological studies. Now, with improved methods to label and monitor inhibitory synapses *in vivo*, researchers have found a surprising degree of dynamics in inhibitory synapses and its potential role in regulating network activity within complex plastic events. In response to the chronic reduction or elevation of input activity, in addition to the homeostatic regulation of the principle neuron itself, the inhibitory neuron in the network can modify its connectivity to both the excitatory input and postsynaptic principle neurons, and contribute to counterbalancing the increased network activity (**Figure 3**; Turrigiano and Nelson, 2004). The inhibitory input within the network can, therefore, function as a perfect site for actions of homeostatic plasticity (Turrigiano and Nelson, 2004; Butz et al., 2009a; Turrigiano, 2012). This theory is strongly supported by previous experiments demonstrating functional homeostasis in a network (Hensch et al., 1998; Rutherford et al., 1998; Desai et al., 2002; Kilman et al., 2002), while emerging evidence also demonstrate that reorganization and changes in the dynamics of inhibitory synapses might contribute to network stabilization.

In the mouse barrel cortex, prolonged single whisker stimulation led to elevated neuronal activity and a transient increase of spine numbers in the corresponding cortical barrel. Subsequently, total synaptic density returned to pre-stimulation levels while only the inhibitory GABA synapses were maintained, potentially compensating for increased sensory input through elevated inhibitory input in the circuit (Knott et al., 2002). Two recent studies specifically labeled and studied cortical inhibitory interneurons in response to sensory deprivation. In the mouse visual cortex, neuropeptide Y (NPY)- positive inhibitory interneurons in layers 1 and 2/3 receive glutamatergic excitatory input on dendritic spines, while sending inhibitory signals to excitatory neurons through axonal boutons (Keck et al., 2011). Seventy-two hours after focal retinal lesions, the spine number in these inhibitory neurons was significantly reduced in the LPZ. In parallel, the number of their axonal boutons also decreased, suggesting a lower level of inhibition was induced by the loss of excitatory input in the network. Another study focused on layer 2/3 interneurons in the visual cortex, where binocular deprivation specifically increased retractions of the branch tips, while the monocular deprivation induced dynamic dendritic arbor rearrangements and reduced axonal bouton numbers onto layer 5 pyramidal apical dendrites (Chen et al., 2011). In both cases, the structural changes in inhibitory neurons could lead to reduction of overall inhibitory drive and serve as a part of the homeostatic response toward sensory deprivation.

The dendritic spines of cortical pyramidal neurons contain both excitatory synapses receiving excitatory input from intracortical axons and thalamocortical axons, as well as inhibitory synapses from local interneurons (Kubota et al., 2007; Gambino and Holtmaat, 2012). Using fluorescent-tagged gephyrin as a marker for inhibitory synapses, two groups studied inhibitory synapses dynamics in excitatory cortical layer 2/3 pyramidal neurons through long-term *in vivo* imaging (Chen et al., 2012; van Versendaal et al., 2012). Both studies made similar observations that a high fraction of gephrin-labeled synapses, ∼30–40%, are localized on dendritic spines, and that they are highly dynamic. Interestingly, a short period of monocular deprivation (1–4 days) caused a significant increase in eliminating inhibitory synapses and a decrease in adding newly formed inhibitory synapses, especially those present on dendritic spines. This large-scale pruning of the inhibitory synapses could lead to increased excitability and constitute a homeostatic response of pyramidal neurons to compensate for the loss of excitatory input.

IMPLICATIONS OF STRUCTURAL HOMEOSTASIS IN PHYSIOLOGICAL AND PATHOLOGICAL CONDITIONS OF THE BRAIN

Proper establishment and maintenance of functional circuits rely on the abilities of neural networks to adjust their excitability based on the input they received. The deficits in compensatory structural reorganization during development or in adulthood have been implicated in a number of brain disorders. Clinical pathology studies link abnormality in dendrite morphology and neuronal homeostasis with several types of neurodevelopmental disorders and psychiatric diseases (Ramocki and Zoghbi, 2008; Toro et al., 2010; Arguello and Gogos, 2012). Mutations in genes regulating synaptogenesis and neuronal circuit formation have been associated with the increased risk of mental illnesses (Toro et al., 2010; Wondolowski and Dickman, 2013). Moreover, structural alterations in specific neural circuits are observed in patients with chronic stress and depression (Castrén and Hen, 2013). Although the causal relationship

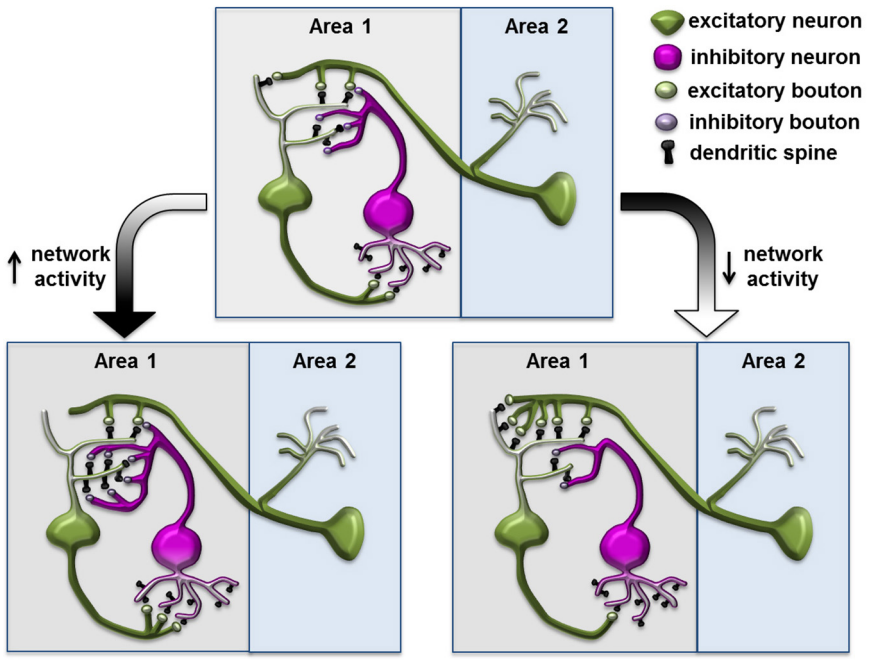

FIGURE 3 | Structural plasticity of inhibitory synapse in a neuronal network during homeostasis. Increased network activity leads to reduced postsynaptic density on the excitatory neuron and increased synapses and spouting on the inhibitory neuron through connections to the excitatory neuron; conversely, decreased network activity leads to increased postsynaptic density on the excitatory neuron and reduced inhibitory elements on the inhibitory neuron. This diagram is based on the homeostatic network theory and is inspired by the schematics from Turrigiano and Nelson (2004), Butz et al. (2009b) and Gambino and Holtmaat (2012).

between anatomical changes and pathological conditions has not yet been established, it is likely that the deficits in structural plasticity and neuronal homeostasis contribute to neurological symptoms. Findings generated using new paradigms and model systems that we described above will undoubtedly update our views on the wiring dynamics of the nervous system and provide us clues to better understand these neurological disorders.

There are also emerging links between neuronal homeostasis and physiological functions of the brain. A particularly noteworthy area of research links sleep, an essential part of animal physiology, with synaptic homeostasis. Despite biological and clinical significance and decades of intense research, the function of sleep remains elusive. A hypothesis proposed by Tonini and Cirelli suggested that the main purpose of sleep is to produce global weakening of synaptic connections that were added or strengthened through experience and learning during the waking period (Tononi and Cirelli, 2003, 2006). The connection between sleep and synaptic homeostasis is supported by experiments carried out in *Drosophila*, where sleep was associated with widespread reduction in synapse number and level of molecules functioning as critical synaptic components (Bushey et al., 2011). In addition, there is evidence suggesting that the structural synaptic plasticity in the zebrafish circadian circuit is under both circadian and sleep-related homeostatic regulation. Sleep deprivation leads to increased synapse number along the axons projecting to the target tissue (Appelbaum et al., 2010). Although structural alterations in large areas of the brain occurring during sleep are yet to be confirmed in mammals, the concept of global synaptic downscaling during sleep is supported by recent findings in mammalian systems (Chauvette et al., 2012; Grosmark et al., 2012). This intriguing hypothesis indeed presents a possible explanation for the necessity of sleep in all animals, which is to ensure neuronal homeostasis and allow the Hebbian form of plasticity to occur daily throughout life.

CONCLUSIONS

Studies we discussed here support structural homeostasis as an important component of neuronal plasticity and open up new areas for future investigations. At the same time, they add to the existing complexity of the array of mechanisms regulating neuronal form and function in the plastic events that lead to adaptation and learning. Alterations in morphology and connectivity are powerful ways to react to the sustained change in global activity, consolidate modifications in synaptic strength and efficacy, and strongly influence subsequent functional and behavioral adaptations. The capacity for structural homeostasis, therefore, could potentially serve as the target for regulatory mechanisms that shift the balance between wiring stability and flexibility within specific circuits and developmental stages.

Many questions remain unanswered in terms of the function and mechanism of structural homeostasis and its interactions with other forms of plasticity. For example, how do synapses,

neurons or circuits sense the activity perturbation and initiate structural modification during homeostasis? What is the sequence of events and their temporal scale? Are there shared cellular and molecular pathways among structural and functional homeostasis, and Hebbian form plasticity? And how do they cooperate with development and functional adaptation? New experimental evidence obtained through updated technologies, such as simultaneous structural and functional imaging in behaving animals, as well as the reexamination of classic paradigms within the new context will both help us address these issues and improve our understanding of neuronal plasticity as a unity.

ACKNOWLEDGMENTS

We thank Uzma Javed, Bo Qin and Devee Schoenberg for the help with the preparation of the manuscript. This work is supported by the intramural research program of the National Institute of Neurological Disorders and Stroke.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 06 September 2014; accepted: 05 December 2014; published online: 20 January 2015.

Citation: Yin J and Yuan Q (2015) Structural homeostasis in the nervous system: a balancing act for wiring plasticity and stability. Front. Cell. Neurosci. ***8****:439. doi: 10.3389/fncel.2014.00439*

This article was submitted to the journal Frontiers in Cellular Neuroscience.

frontiers in
CELLULAR NEUROSCIENCE

REVIEW ARTICLE
published: 21 November 2013
doi: 10.3389/fncel.2013.00219

Activity-dependent adaptations in inhibitory axons

Cátia P. Frias and Corette J. Wierenga*

Division of Cell Biology, Faculty of Science, Utrecht University, Utrecht, Netherlands

Edited by:
Jaichandar Subramanian, Massachusetts Institute of Technology, USA

Reviewed by:
Arianna Maffei, Suny Stony Brook, USA
Andreas Vlachos, Goethe University Frankfurt, Germany

***Correspondence:**
Corette J. Wierenga, Division of Cell Biology, Faculty of Science, Utrecht University, Padualaan 8, 3584 CH Utrecht, Netherlands
e-mail: c.j.wierenga@uu.nl

Synaptic connections in our brains change continuously and throughout our lifetime. Despite ongoing synaptic changes, a healthy balance between excitation and inhibition is maintained by various forms of homeostatic and activity-dependent adaptations, ensuring stable functioning of neuronal networks. In this review we summarize experimental evidence for activity-dependent changes occurring in inhibitory axons, in cultures as well as *in vivo*. Axons form many presynaptic terminals, which are dynamic structures sharing presynaptic material along the axonal shaft. We discuss how internal (e.g., vesicle sharing) and external factors (e.g., binding of cell adhesion molecules or secreted factors) may affect the formation and plasticity of inhibitory synapses.

Keywords: GABAergic synapses, interneurons, homeostatic plasticity, axons, cell adhesion molecules

INTRODUCTION

Our brain is a complex organ with tremendous self-organizing abilities. Its computational power is based in the adjustable synaptic connections between neurons. When new experiences and memories are established, specific synapses in specific brain regions are changed, both in strength and in number. To ensure proper global functioning despite changes in local connectivity, these synaptic changes must be coordinated within neurons, as well as within neuronal circuits. An important aspect is the coordination between changes in excitatory and inhibitory synapses to regulate and maintain an overall balance between excitation and inhibition. When this balance is disturbed, neurological diseases such as autism or schizophrenia can develop (Palop et al., 2007; Yizhar et al., 2011; Han et al., 2012; Bateup et al., 2013).

Homeostatic plasticity is a term that is used for plasticity mechanisms which ensure that overall neuronal spiking activity is maintained within neuronal networks. Many forms of homeostatic plasticity have been described in excitatory and inhibitory neurons (Turrigiano, 2008; Wenner, 2011; Pozo and Goda, 2010; Tyagarajan and Fritschy, 2010). In neuronal circuits in the brain, inhibitory neurons serve multiple functions, making connections to excitatory as well as inhibitory neurons, and providing feedforward inhibition to some neurons, while supplying feedback input to others. In such complicated networks, there are multiple ways to compensate for changes in network activity, which makes it hard, if not impossible, to classify synaptic changes in inhibitory axons as truly homeostatic. Therefore, we will discuss activity-dependent feedback signals in inhibitory axons in a more general context in this review. We will discuss experimental evidence showing that synaptic activity can affect the formation and plasticity of inhibitory synapses and we will speculate on possible mechanisms.

ACTIVITY-DEPENDENT ADAPTATIONS OF INHIBITORY SYNAPSES

When prolonged changes occur in network activity, homeostatic mechanisms come into play which adjust excitatory and inhibitory synapses to compensate and restore the activity level in the network (Turrigiano, 1999, 2011; Burrone and Murthy, 2003; Pozo and Goda, 2010; Wenner, 2011). Generally speaking, when the activity is too high, excitation must be downregulated, and inhibition should be increased to bring activity levels back to baseline. And opposite changes should occur during activity blockade. Homeostatic plasticity has been studied extensively in cultures, where neurons are randomly connected. Dissociated cultures provide superb access for experimental manipulations and therefore form an excellent system to study the cell biological mechanisms underlying homeostatic plasticity. However, in our brain neurons are embedded in multiple neuronal networks and make specific synaptic connections. Recurrent connections between neurons or groups of neurons are very common and different types of GABAergic interneurons are known to have high specificity, making inhibitory synapses onto specific target neurons, including inhibitory neurons (Pfeffer et al., 2013; Jiang et al., 2013). In such complex networks, it is not easy to determine rules of homeostatic plasticity. Adaptation to changes in the activity of the network will be strongly synapse-specific and likely depends on the precise function and location of the synapse in the network (Chen et al., 2011; Maffei et al., 2004; Maffei and Turrigiano, 2008). Here we briefly describe the experimental evidence for activity-dependent plasticity of inhibitory synapses from *in vitro* (i.e., in dissociated and organotypic cultures) and *in vivo* studies.

Primary cell cultures

Activity manipulations in cultures of dissociated hippocampal or neocortical neurons generally affect excitatory and inhibitory synapses in opposite directions. After a prolonged period of activity blockade, excitatory synapses get strengthened and inhibitory synapses are weakened, and synaptic changes are in opposite directions when activity is enhanced (Turrigiano et al., 1998; Kilman et al., 2002; Hartman et al., 2006; Swanwick et al., 2006). Therefore, changes in excitation and inhibition cooperate to compensate for the change in activity level. For inhibitory synapses, changes

in mIPSC amplitude are most commonly reported, reflecting changes in synaptic strength. Sometimes they are accompanied by changes in mIPSC frequency, which could either reflect a change in the number of synapses or a change in release properties. Dissociated cultures provide excellent experimental access and are therefore well-suited for studying underlying mechanisms of homeostatic plasticity. However, the artificial environment in which neurons grow in culture may affect synaptic maturation (Wierenga et al., 2006; Rose et al., 2013) and consequently cellular or synaptic mechanisms of plasticity. Cellular mechanisms that were identified to mediate the changes in inhibitory synapses after activity manipulations include: changes in number of postsynaptic receptors (Kilman et al., 2002; Swanwick et al., 2006; Saliba et al., 2007; Peng et al., 2010; Rannals and Kapur, 2011) or scaffolding proteins (Vlachos et al., 2012; study in slice cultures) on the postsynaptic side, and changes in presynaptic release probability (Kim and Alger, 2010), presynaptic vesicle loading (De Gois et al., 2005; Hartman et al., 2006; Lau and Murthy, 2012), or GABA-producing enzymes (Peng et al., 2010; Rannals and Kapur, 2011) on the presynaptic side. Only in a few cases, changes in the number of inhibitory synapses were reported (Hartman et al., 2006; Peng et al., 2010). Homeostatic changes of inhibitory synapses could be induced in a cell autonomous fashion (Peng et al., 2010), or required a change in activity of the entire neuronal network (Hartman et al., 2006), emphasizing that there are multiple mechanisms of homeostatic plasticity at inhibitory synapses. In particular, distinct mechanisms could exist for activity-dependent downregulation and upregulation of inhibitory synapses.

Organotypic cultures

In contrast to dissociated cultures neurons in more intact tissue, such as acute slices or organotypic cultures, make more specific connections and form structured networks. This network configuration makes the interpretation of synaptic changes more complex. In slices that were submitted to activity manipulations, changes in inhibition have been observed opposite to (Marty et al., 2004; Karmarkar and Buonomano, 2006; Kim and Alger, 2010) as well as in conjunction with (Buckby et al., 2006; Echegoyen et al., 2007) changes in excitation. It was also shown that different types of homeostatic mechanisms have different time courses (Karmarkar and Buonomano, 2006) and that different subsets of inhibitory synapses can respond differently. For instance, the presence of cannabinoid receptors in a subset of inhibitory synapses renders them selectively receptive to changes in endocannabinoid levels induced by inactivity (Kim and Alger, 2010). In another example, inactivity differentially affected somatic and dendritic inhibitory inputs on CA1 pyramidal cells. Interestingly, both types of synapses showed reduction in the number of presynaptic boutons and upregulation of release probability, but the functional end-effect on inhibitory input to the postsynaptic cells was different (Chattopadhyaya et al., 2004; Bartley et al., 2008). This emphasizes that simple *in vitro* homeostatic rules for scaling inhibitory synapses get complicated in more complex networks. In addition, other factors such as different cell (glia) types or the extracellular environment in more intact tissue potentially influence homeostatic plasticity compared to dissociated cultured cells.

In vivo studies

Typically, when studying activity-dependent or homeostatic changes *in vivo*, sensory deprivation is used as experimental paradigm to lower activity levels in the primary sensory cortex (e.g., whisker trimming, monocular deprivation, or retinal lesion). While *in vitro* activity manipulations by pharmacological means affect the activity of all neurons in equal amounts, sensory deprivation *in vivo* will affect different neurons in the circuitry differentially. Therefore, *in vivo* responses of inhibitory synapses to changes in activity vary widely and strongly depend on the specific cell types, cortical layer, and specific circuitry (Maffei et al., 2004; Maffei and Turrigiano, 2008; Chen et al., 2011). Furthermore, it is well-known that inhibition in sensory cortex areas undergoes important developmental changes (Hensch, 2005), which means that the same deprivation paradigm can have different effects on inhibitory synapses depending on the postnatal period that is considered (Chattopadhyaya et al., 2004; Maffei et al., 2006; Maffei et al., 2010). An emerging theme from the *in vivo* studies is that inhibitory synapses can respond rapidly to sensory deprivation. It was shown that inhibitory axons in cortical layer 2/3 reduce the number of boutons within the first 24 h after a retinal lesion or monocular deprivation (Chen et al., 2011; Keck et al., 2011). Over longer periods, inhibitory axons in the barrel cortex were shown to sprout and form new axonal branches after whisker plucking (Marik et al., 2010). Interestingly, the reduction of inhibition was often found to precede adaptive changes of the excitatory circuitry (Marik et al., 2010; Keck et al., 2011). The rapid downregulation of inhibition might serve to render the local circuit more permissive for excitatory plasticity to occur (Ormond and Woodin, 2011; Gambino and Holtmaat, 2012). In two recent studies it was shown that inhibitory synapses that are located on spines (presumably next to an excitatory synapse) showed much higher turnover rates compared to inhibitory synapses on shaft after visual deprivation (Chen et al., 2012; vanVersendaal et al., 2012). It will be interesting to see whether direct cross talk of the two types of synapses exists.

In conclusion, there is a large amount of compelling evidence for activity-dependent adaptations in inhibitory synapses *in vitro* as well as *in vivo*. The precise expression mechanisms significantly vary between different preparations and experimental paradigms.

AXONS

In this review we focus on possible feedback signals that occur in inhibitory axons in response to changes in network or synaptic activity and that induce changes in the number or properties of presynaptic terminals along the axon. The axon of a single neuron forms several thousands of presynaptic terminals (i.e., "boutons") along its shaft and contacts many different postsynaptic neurons. Presynaptic boutons along an axon show a large variety in their volumes, in the number of synaptic vesicles and in the presence or absence of mitochondria (Shepherd and Harris, 1998). It is now well-established that neighboring boutons are not independent entities, but they continuously share and exchange molecular components of the release machinery and synaptic vesicles (Krueger et al., 2003; Darcy et al., 2006; Sabo et al., 2006; Staras, 2007; Yamada et al., 2013). Synaptic vesicles may not belong to a specific presynaptic terminal, but form a super pool of vesicles in

the axonal shaft and are shared by multiple release sites (Staras et al., 2010).

The exchange of presynaptic proteins means that the exact composition of presynaptic terminals is continuously changing. These changes can occur in a correlated fashion with the postsynaptic site in some synapses, but can be uncoordinated in others (Fisher-Lavie et al., 2011; Fisher-Lavie and Ziv, 2013). Release properties and synaptic strength are highly variable between individual boutons along the same axon (Branco et al., 2008; Zhao et al., 2011; Rose et al., 2013). Therefore the demand for synaptic vesicles or other presynaptic proteins will vary between presynaptic boutons and neighboring boutons compete for available resources. Indeed, reduced availability of synaptic proteins within the axon has been shown to enhance competition between boutons (Yamada et al., 2013). In addition, vesicle exchange is regulated by neuronal activity through changes in axonal calcium levels (Kim and Ryan, 2013, 2010).

Synaptic vesicles are kept at the presynaptic terminal by interacting with a scaffolding meshwork of actin, β-catenin, synapsin, and many other proteins (Bamji et al., 2003; Takamori et al., 2006; Cingolani and Goda, 2008; Fernández-Busnadiego et al., 2010; Peng et al., 2012; Taylor et al., 2013). Synaptic vesicles can escape from the presynaptic terminal into the axon, while other vesicles that were traveling along the axonal shaft can be captured. Although the loss of a strict presynaptic compartmentalization may seem disadvantageous at first, the main advantage of sharing presynaptic material between boutons is flexibility. When presynaptic material is continuously being lost and gained at synapses, synapses can rapidly change their strength by adjusting the ratio of vesicle capture and release (Wu et al., 2013). In addition, synapses can be formed or disassembled within a few hours. It was shown that presynaptic proteins can be transported together in small packages in axons (Friedman et al., 2000; Zhai et al., 2001; Wu et al., 2013). Such multi-protein packages can be recruited to locations where new synapses are being formed and a few of these ready-to-go packages are enough to rapidly build a functional active zone and release site (Jin and Garner, 2008; Owald and Sigrist, 2009).

Live imaging of axons have shown that transient and mobile release sites exist (Krueger et al., 2003) and that transient boutons occur at predefined locations along the axon (Sabo et al., 2006; Ou and Shen, 2010; Bury and Sabo, 2011), presumably reflecting contact sites with potential postsynaptic targets (Wierenga et al., 2008; Schuemann et al., 2013). The transient nature of boutons in such locations suggest that presynaptic structures are immature or incomplete and may serve a role in "testing" a new synaptic location (Wierenga et al., 2008; Dobie and Craig, 2011; Fu et al., 2012; Schuemann et al., 2013). Transient boutons might therefore reflect failed attempts or intermediate stages of building new synapses, but they could also have a physiological function. Transient boutons, or orphan release sites, are likely capable of neurotransmitter release (Krueger et al., 2003; Coggan et al., 2005; Ratnayaka et al., 2011) and besides having a role in synapse formation, ectopic release of neurotransmitter by transient boutons could also serve to signal to nearby astrocytes or to regulate ambient neurotransmitter levels.

Synapse assembly is a complicated process involving interactions of multiple proteins. It does not necessarily need to be a linear process, where one component necessarily recruits the next, but some of the interactions could occur in parallel and the sequence of protein recruitment may vary. Rapid self-assembly of presynaptic components may be an important element during synaptogenesis. This would mean that the formation of a presynaptic terminal merely needs an initial trigger to ascertain a specific axonal location or postsynaptic partner, but then the new presynaptic terminal "unfolds" automatically by spontaneous clustering of its components. It is likely that multiple triggers can induce self-assembly. Indeed, it was recently reported that synaptic material is actively prevented from aggregating and assembling new synapses during transport (Wu et al., 2013), supporting the self-assembly hypothesis. Without prevention of aggregation, presynaptic terminals were formed at locations where no postsynaptic targets were present and no postsynaptic specializations were recruited. Furthermore, the ectopic formation of presynaptic terminals on non-neuronal cells can be induced when these cells express "synaptogenic" cell adhesion molecules (Scheiffele et al., 2000; Graf et al., 2004; Takahashi et al., 2012), indicating that a single trans-synaptic trigger is enough to start the presynaptic cascade to assemble functional release sites.

A dynamic control of the strength and number of presynaptic terminals in axons implies that control of transport, capture, and release of synaptic material are essential processes regulating the formation, maintenance, and strength of presynaptic terminals. In a dynamic axon with competing presynaptic terminals, a general change in synaptic strength is expected to also have an effect on ongoing synapse formation within the same axon and vice versa (**Figure 1**). For instance, enhancement of synaptic strength by increasing vesicle capture or anchoring at presynaptic terminals would also result in lower amounts of "free" vesicles in the axonal shaft thereby reducing the chance that new synapses are formed at nascent sites (Yamada et al., 2013). However, a similar increase in synaptic strength could also be achieved by increasing vesicle clustering (Wu et al., 2013), but such a mechanism would actually promote synapse formation (**Figure 1**). This illustrates that presynaptic plasticity and synapse formation should be considered mutually dependent processes when neighboring presynaptic terminals are sharing synaptic proteins and vesicles.

INHIBITORY AXONS

Most of the studies that were mentioned above were performed in excitatory axons and it is not entirely clear to what extent the results are also valid for inhibitory axons. Important observations have been made in live imaging studies of inhibitory axons. Presynaptic terminals in inhibitory axons were shown to be dynamic structures *in vitro* and *in vivo*. Inhibitory boutons can appear, disappear, and reappear over the course of several minutes to hours (Kuhlman and Huang, 2008; Marik et al., 2010; Keck et al., 2011; Fu et al., 2012; Schuemann et al., 2013), and the same has been shown for clusters of pre- or postsynaptic proteins at inhibitory synapses (Dobie and Craig, 2011; Chen et al., 2012; Kuriu et al., 2012; van-Versendaal et al., 2012). Bouton dynamics are comparable *in vitro* and *in vivo* and likely reflect physiological processes. Interestingly, these dynamic changes were shown to be affected by network activity and mediated, at least in part, by activation of GABA receptors

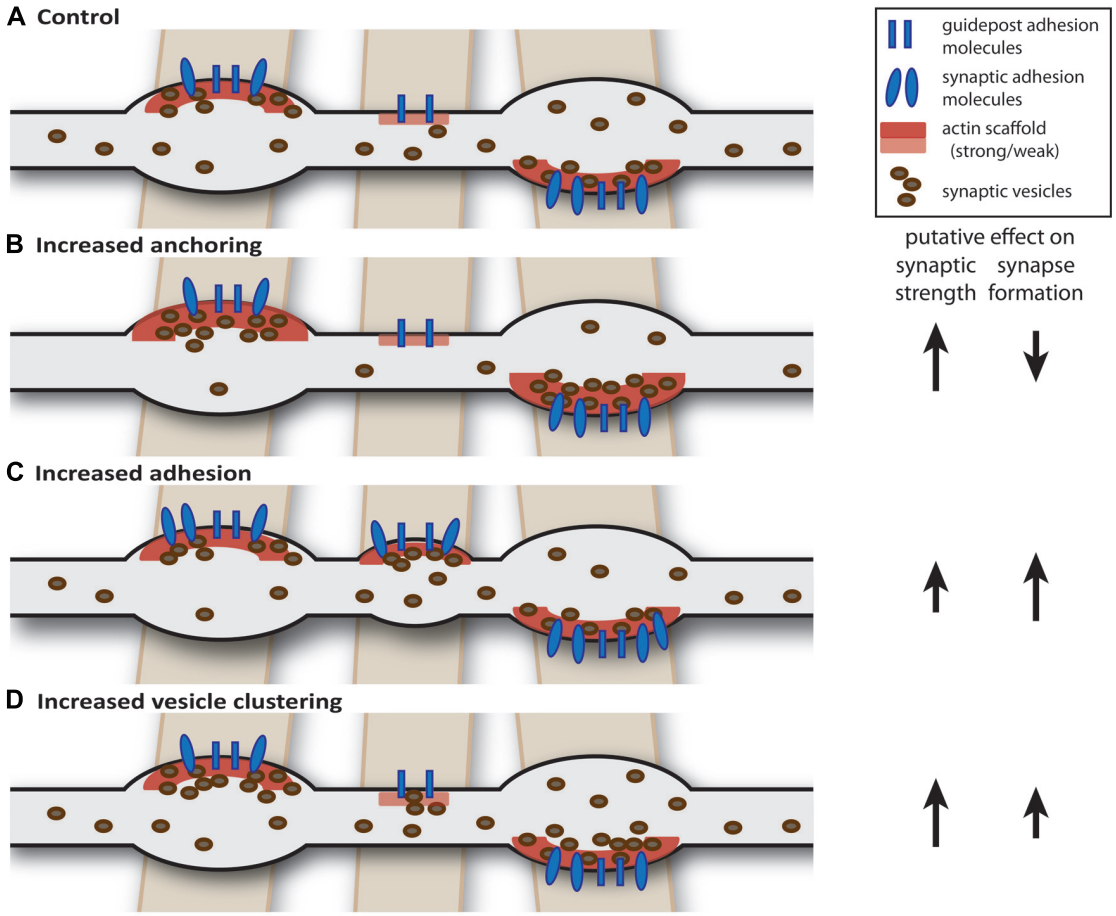

FIGURE 1 | Intrinsic factors: axon-wide increase in synaptic strength or release properties may also affect synapse formation. (A) Schematic drawing of an axon (gray) forming two mature and one nascent bouton on crossing dendrites (brown). We hypothesize that axon-dendrite crossings are marked at potential synaptic locations and contain guidepost adhesion molecules (Shen and Bargmann, 2003; Shen et al., 2004) and weak actin scaffold (Chia et al., 2012). **(B)** Increasing anchoring of vesicles at presynaptic terminals could decrease the pool of "free" vesicles, thereby reducing the probability of forming new synapses (Yamada et al., 2013). **(C)** Increasing synaptic adhesion increases the number of synapses (Scheiffele et al., 2000; Takahashi et al., 2012; Kuzirian et al., 2013) and may also affect properties of existing synapses (Varoqueaux et al., 2006; Wittenmayer et al., 2009). **(D)** Overexpression of vesicle clustering factors induce changes in release properties, but may also promote synapse formation (Wentzel et al., 2013; Wu et al., 2013).

(Fu et al., 2012; Kuriu et al., 2012; Schuemann et al., 2013). This could represent a mechanism by which the synaptic activity of inhibitory synapses may regulate their own stability using GABA as a feedback signal.

New inhibitory synapses can form rapidly by the appearance of a bouton at locations where the inhibitory axon is in close contact with a dendrite, without the involvement of dendritic protrusions (Wierenga et al., 2008; Dobie and Craig, 2011). This finding indicates an important contrast with the formation of excitatory synapses, in which new synapses are usually formed by the outgrowth of dendritic protrusions. It also emphasizes the important role of crosstalk between neighboring boutons within inhibitory axons for synapse formation. Nascent inhibitory synapses recruit release machinery proteins and synaptic vesicles on the presynaptic side and receptors and scaffolding molecules on the postsynaptic side within a few hours (Wierenga et al., 2008; Dobie and Craig, 2011; Kuriu et al., 2012; Schuemann et al., 2013). Interestingly, simultaneous translocations of pre- and postsynaptic proteins over several micrometers were observed in cultures (Dobie and Craig, 2011; Kuriu et al., 2012) and it will be interesting to see if such movement of inhibitory synapses can also occur in slices or *in vivo*. Together, these observations reveal the dynamic nature of inhibitory axons and strongly suggest that the exchange of presynaptic material between existing and emerging boutons within the axonal shaft plays an essential role in the activity-dependent formation, maintenance and plasticity of inhibitory synapses.

In general, it is not clear if molecular differences exist between excitatory and inhibitory axons, other than the neurotransmitter that is produced and loaded into synaptic vesicles. For instance, the extent or regulation of dynamic exchange between boutons could be different in these two types of axons. The protein composition of the release machinery at excitatory and inhibitory presynaptic terminals is surprisingly similar, although small difference have been reported (Gitler et al., 2004; Kerr et al., 2008; Kaeser et al.,

2009; Grønborg et al., 2010; Zander et al., 2010; Boyken et al., 2013; Bragina et al., 2013). It is currently not known if some of these differences have consequences for plasticity or presynaptic dynamics within axons. Furthermore, it is not known if there are differences between axons of the various inhibitory cell types (Ascoli et al., 2008; Klausberger and Somogyi, 2008). However, there is a clear difference between excitatory and inhibitory axons in the expression of specific cell adhesion molecules at excitatory and inhibitory synapses.

ROLE OF CELL-ADHESION MOLECULES IN SYNAPTIC PLASTICITY

The observation that inhibitory boutons appear at specific, predefined locations along the axon (Sabo et al., 2006; Wierenga et al., 2008; Schuemann et al., 2013), strongly suggests that something is marking these locations prior to bouton formation (Shen and Bargmann, 2003; Shen et al., 2004). Inhibitory axons are characterized by their tortuous and highly branched morphology and they are in close contacts with many nearby dendrites. In fact, it was shown that inhibitory axons have substantially larger overlap with the dendritic trees of their potential target neurons than expected from chance, whereas this is not the case for excitatory axons (Stepanyants et al., 2004). This suggests that inhibitory axons possibly search for or are attracted by dendrites during development. Contacts between dendrites and inhibitory axons could be maintained by guidepost cell-adhesion molecules, even without inhibitory synapses present (Shen and Bargmann, 2003; Shen et al., 2004). Their presence would mark the location of a postsynaptic dendrite and therefore a potential spot for an inhibitory synapse.

Cell adhesion molecules are transmembrane proteins, which play a role in recognition of synaptic partners during the initial contact and provide specificity of synaptic connections (Meijers et al., 2007; Wojtowicz et al., 2007). In addition, cell adhesion molecules have been shown to play a role in the process of synaptic maturation following the initial contact, in the recruitment of synaptic proteins as well as in maintaining proper synaptic function throughout the lifetime of the synapse (Dalva et al., 2007; Krueger et al., 2012; Thalhammer and Cingolani, 2013). Cell adhesion molecules can also play an active role in the process of synapse disassembly (O'Connor et al., 2009). In conclusion, cell adhesion molecules are an essential part of synapses and synaptic plasticity most likely involves regulation of cell-adhesion molecules. Here we discuss how synaptic adhesion could be regulated in an activity-dependent manner (**Figure 2**) and we summarize current knowledge of cell adhesion molecules that are specific for inhibitory synapses.

Activity-dependent regulation of protein expression levels

Cell adhesion molecules often serve as recognition or identity signals to specify neuronal connectivity, and they can either promote or prevent synapse formation (Dalva et al., 2007; Bukalo and Dityatev, 2012). Neurons presumably express a combination of cell adhesion molecules and the specific combination (both the variety as well as relative levels) likely regulate the specificity and number of their synaptic contacts (Sassoè-Pognetto et al., 2011). Different cell adhesion molecules can cooperate to promote synapse formation, but the opposite is also possible: cis-interactions between different cell adhesion molecules within a neuron can preclude trans-interactions with cell adhesion molecules on neighboring neurons and thereby inhibit or prevent synapse formation (Taniguchi et al., 2007; Lee et al., 2013). Most importantly, the combination of cell adhesion molecules that a neuron expresses might not be static (**Figure 2A**). Indeed, for a number of cell adhesion molecules, activity-dependent changes in expression level have been observed (Pinkstaff et al., 1998; Cingolani et al., 2008; Pregno et al., 2013). Changes in expression level may be regulated by the activity level of the neuron itself or by extracellular signals from the environment, such as secreted factors from neighboring cells. For instance, TNFα, a glia-derived factor, which is secreted in an activity-dependent manner, regulates expression levels of β3 integrin and N-cadherin (Kubota et al., 2009; Thalhammer and Cingolani, 2013). In theory, local protein synthesis in the axon could also contribute to changes in expression level of cell adhesion proteins (Taylor et al., 2009, 2013; Zivraj et al., 2010), but direct experimental evidence is currently lacking.

Activity-dependent regulation of splicing

For many adhesion molecules different splice forms have been identified. Different splice variants often have different affinities for their binding partners and thereby differentially affect synapse formation or plasticity (Missler and Südhof, 1998; Hattori et al., 2008; Aoto et al., 2013). For instance, alternative splicing of neuroligins and neurexins affects specificity for excitatory or inhibitory synapses (Chih et al., 2006; Graf et al., 2006). Therefore, alternative splicing might be a way to enlarge the available set of adhesion molecules within a neuron and to enhance the range of molecular specificity of synaptic connections.

Activity-dependent regulation of cell surface distribution

To have their effect specifically at synapses, cell adhesion molecules should be enriched at synaptic membranes. There is experimental evidence that the distribution of cell adhesion molecules over the cellular surface can be regulated (Tai et al., 2007; Fu and Huang, 2010). For instance, while neurexin1α shows a diffuse pattern along the axonal membrane in inhibitory axons, neurexin1β is specifically enriched in the membrane at presynaptic terminals. Anchoring of neurexin1β at presynaptic boutons is regulated by presynaptic GABA release and subsequent $GABA_B$ receptor activation (Fu and Huang, 2010). Further investigation is needed to understand how such local changes are regulated by protein modifications or localized endo- or exocytosis and how they affect local synapse formation (**Figure 2B**).

Activity-dependent regulation of protein cleavage

Synaptic adhesion molecules execute their function by binding to a trans-synaptic partner at their extracellular domain. In some cases, the extracellular domain can be cleaved, with strong effects on local synaptic adhesion. For instance, activity-dependent cleavage of agrin was shown to mediate the formation of dendritic filopodia (Frischknecht et al., 2008; Matsumoto-Miyai et al., 2009) and cleavage of neuroligin-1 was shown to regulate synaptic strength of individual excitatory synapses in an activity-dependent manner (Peixoto et al., 2012; Suzuki et al., 2012). Many other adhesion molecules have known cleavage sites and it will be interesting to

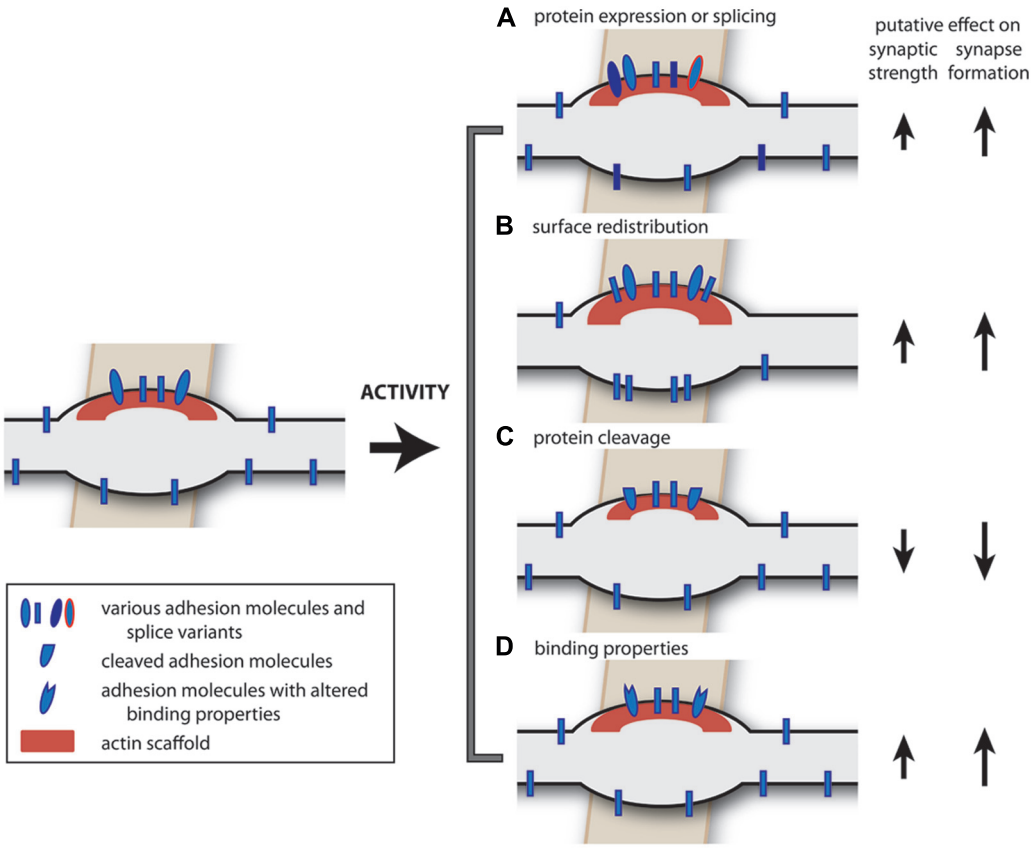

FIGURE 2 | Extrinsic factors: possible activity-dependent changes in cell adhesion molecules. Neural activity may induce a number of changes in adhesion molecules. **(A)** The expression level of cell adhesion molecules (Cingolani et al., 2008), or their splice variants (Chih et al., 2006; Graf et al., 2006), can be regulated in an activity-dependent manner, potentially affecting synapse formation, and synapse specificity. **(B)** Activity-dependent redistribution of adhesion molecules over the axonal membrane can facilitate synapse formation (Fu and Huang, 2010). **(C)** Activity-dependent cleavage of synaptic adhesion molecules could induce synapse disassembly or plasticity (Matsumoto-Miyai et al., 2009; O'Connor et al., 2009; Peixoto et al., 2012; Suzuki et al., 2012). **(D)** Activity-dependent changes in binding properties of adhesion molecules (Kim et al., 2011a,b) could affect synaptic properties. In addition, the intracellular signaling pathways (not depicted) may also be regulated in an activity-dependent manner, affecting all of these processes.

see whether this mechanism for activity-dependent regulation is also present at inhibitory synapses (**Figure 2C**).

Activity-dependent regulation of binding

For some cell adhesion molecules activity can regulate the binding properties of the proteins. For instance, interactions between cadherins are affected by extracellular calcium concentrations (Kim et al., 2011b) and integrins can switch between an active or inactive configuration by extra- or intracellular factors (Hynes, 2002). In this way, synaptic adhesion can be modulated in an activity-dependent manner without a change in synaptic composition (**Figure 2D**).

Activity-dependent regulation of interacting proteins

Upon binding to other cell adhesion molecules, cell adhesion molecules cluster at the cell membrane and signal through interactions with many intracellular proteins, whose levels may be regulated in an activity-dependent manner. Ultimately, signaling through synaptic adhesion molecules in the presynaptic terminal result in direct or indirect alterations of the actin cytoskeleton and vesicle recycling, affecting the number, function, and/or stability of synapses (Zhang et al., 2001; Tabuchi et al., 2002; Swiercz et al., 2008; Sun and Bamji, 2011; Takahashi and Craig, 2013). It will be crucial to identify the precise molecular pathways that are involved to fully understand how activity-dependent changes at inhibitory synapses occur.

CELL ADHESION MOLECULES AT INHIBITORY SYNAPSES

The list of known synaptic adhesion molecules is rapidly growing, but our knowledge on the precise function of most of these proteins remains incomplete. Interestingly, several synaptic cell-adhesion molecules have been reported to be specifically involved in inhibitory, and not excitatory, synapses. These include sema4D (Paradis et al., 2007; Kuzirian et al., 2013), slitrk3 (Takahashi et al., 2012), and neuroligin-2 (Varoqueaux et al., 2004; Patrizi et al., 2008; Poulopoulos et al., 2009), and it is to be expected that new discoveries will be made in the near future. Here we briefly summarize what is known on the role of various cell adhesion molecules at inhibitory synapses.

NEUROLIGIN-2

Postsynaptic neuroligins and their presynaptic partners neurexins are transmembrane cell adhesion molecules that have been established as important synaptic regulators (Südhof, 2008; Siddiqui and Craig, 2011; Krueger et al., 2012). When expressed in non-neuronal cells, neurexins as well as neuroligins can induce the formation of synapses in co-cultured neurons (Graf et al., 2004; Kang et al., 2008). This suggests that neurexins and neuroligins function in the initial assembly of synaptic connections. However, knock out studies showed that they are not strictly required for synaptogenesis, but they play a crucial role in the proper assembly and functional maturation of synapses (Varoqueaux et al., 2006). Neuroligin-2 localizes specifically to the postsynaptic membrane of inhibitory synapses (Varoqueaux et al., 2004; Chubykin et al., 2007) and has been shown to be a regulator of inhibitory synapse formation and function (Varoqueaux et al., 2006; Chubykin et al., 2007; Poulopoulos et al., 2009). Interestingly, a recent report suggested that the preferential localization of neuroligin-2 at inhibitory synapses can be contributed to the low abundance of β-neurexin1 in inhibitory axons (Futai et al., 2013), suggesting that the presynaptic axon determines specificity of cell adhesion molecules at inhibitory synapses. Mice lacking neuroligin-2 show impairments in inhibitory synaptic transmission and exhibit anxiety-like behavior and increased excitability (Blundell et al., 2009; Gibson et al., 2009; Jedlicka et al., 2011). Interestingly, although neuroligin-2 is present at all inhibitory synapses, only perisomatic synapses were affected in the absence of neuroligin-2 (Gibson et al., 2009). Recently, two adhesion molecules were found to show specific interactions with neuroligin-2 at inhibitory synapses. MDGA1 inhibits the interaction between neuroligin-2 and neurexins and therefore specifically suppresses the inhibitory synaptogenic activity of neuroligin-2 (Lee et al., 2013; Pettem et al., 2013). IgSF9 specifically localizes at inhibitory synapses on inhibitory neurons, where it binds to neuroligin-2 via the scaffolding protein S-SCAM (Woo et al., 2013). These findings raises the possibility that neuroligin-2 serves different functions at different inhibitory synapses, depending on its interactions with other cell adhesion molecules.

SLITRK3

Leucine-rich repeat (LRR) proteins have received considerable research attention recently. The members of the subfamily of Slitrk (Slit and Trk-like) proteins are involved in synapse formation and has been linked to several neurological disorders (Aruga and Mikoshiba, 2003; Takahashi and Craig, 2013). Slitrk3 has been shown to be present at the postsynaptic side of inhibitory synapses and it can induce the formation of inhibitory synapses through its interaction with the presynaptic tyrosine phosphatase receptor PTPδ (Takahashi et al., 2012; Yim et al., 2013). Here, the specificity for inhibitory synapses is dictated by the postsynaptic slitrk3, as it was shown that presynaptic PTPδ can interfere with other synaptic organizing molecules to promote formation of excitatory synapses (Yoshida et al., 2011, 2012). The slitrk3 knock out mouse has no gross defect in brain morphology, but shows decreased expression of inhibitory markers (Takahashi et al., 2012). Accordingly, these mice have an increased susceptibility for seizures and sometimes display spontaneous seizures. Interestingly, not all inhibitory synapses were equally affected by the loss of slitrk3. In the hippocampal CA1 region, specifically inhibitory synapses in the middle of the pyramidal layer were lost (Takahashi et al., 2012). It will be interesting to examine whether specificity of inhibitory synapses correlates with different subsets of pre- or postsynaptic neurons types or function.

Members of the closely related subfamily of leucine-rich transmembrane proteins (LRRTMs) have also been implicated in excitatory synapse formation and plasticity (Linhoff et al., 2009; Ko et al., 2011; de Wit et al., 2013; Siddiqui et al., 2013), but so far no LRRTM that is specific for inhibitory synapses has been identified.

SEMAPHORIN-4D

Semaphorins are well-known as (repulsive) axon guidance molecules acting through rearrangements of the cytoskeleton in the growth cone. They play an important role in the early development of the brain (Pasterkamp, 2012). Some semaphorins are also expressed later in development and have been implicated in the formation and plasticity of neuronal connections (Sahay et al., 2005; Morita et al., 2006; Paradis et al., 2007; O'Connor et al., 2009; Ding et al., 2012; Mizumoto and Shen, 2013). Knocking down the membrane-bound semaphorin Sema4D was shown to specifically reduce the number of inhibitory synapses, while excitatory synapses were not affected (Paradis et al., 2007). Furthermore, application of soluble Sema4D was able to increase the density of GABAergic synapses within 30 min in rat hippocampal neurons (Kuzirian et al., 2013). These new inhibitory synapses became functional within 2 h and could restore normal levels of activity in an *in vitro* model for epilepsy (Kuzirian et al., 2013). The effect of sema4D on inhibitory synapses depends on the plexinB1 receptor (Kuzirian et al., 2013). It was earlier shown that activation of plexinB1 by sema4D can induce opposing responses on the cytoskeleton, depending on different interacting proteins (Basile et al., 2004; Swiercz et al., 2008; Tasaka et al., 2012), but the intracellular pathway used for inhibitory synapse formation is not known. Sema4D is a membrane-bound protein, but the protein can also be cleaved (Basile et al., 2007; Zhu et al., 2007). It was recently shown that extracellular cleavage of sema4D occurs in neurons, but does not interfere with its synaptogenic properties at inhibitory synapses (Raissi et al., 2013).

OTHER CELL ADHESION MOLECULES

There are many other cell adhesion molecule proteins and with continued research on inhibitory synapses, it is expected that more of them will be found to play a role at inhibitory synapses. Here we just mention a few that have been reported at inhibitory synapses.

Neural cell adhesion molecule (NCAM) has been reported to be important for the maturation of perisomatic inhibitory synapses in the cortex (Pillai-Nair et al., 2005; Brennaman and Maness, 2008; Chattopadhyaya et al., 2013). NCAM acts through activation of Fyn kinases and possibly recruits other adhesion molecules (Chattopadhyaya et al., 2013). Interestingly, it was recently reported that also members of the *ephrin* family, ephrinA5 and EphA3, can affect inhibitory synapses and they require NCAM for their action (Brennaman et al., 2013). *In vivo*, NCAM is polysialylated (NCAM-PSA) in an experience-dependent manner

and developmental downregulation of NCAM-PSA was shown to coordinate maturation of perisomatic inhibitory synapses in the visual cortex (Di Cristo et al., 2007).

Several components of the dystrophin-associated glycoprotein complex (DGC), such as dystroglycan, dystrophin, and dystrobrevin, are also specifically located at a subset of inhibitory synapses (Knuesel et al., 1999; Brünig et al., 2002; Lévi et al., 2002; Grady et al., 2006), but the function of this complex at inhibitory synapses is not well understood. The DGC could be linked to postsynaptic neuroligin-2 via the scaffolding protein S-SCAM (Sumita et al., 2007) and to presynaptic neurexins (Sugita, 2001). Interestingly, a synaptic guanine exchange factor SynArfGEF has been identified that specifically co-localizes at inhibitory synapses, which could be involved in the downstream signaling pathway of the DGC (Fukaya et al., 2011), but its exact function remains to determined.

Integrins are receptors for extracellular matrix proteins, soluble factors, and counter-receptors on adjacent cells and they have an intracellular link to actin filaments via adaptor proteins (Hynes, 2002; Harburger and Calderwood, 2009). Integrins have been implicated in activity-dependent synaptic changes (Chavis and Westbrook, 2001; Chan et al., 2003) and in homeostatic scaling of excitatory synapses (Cingolani et al., 2008). At glycinergic inhibitory synapses in the spinal cord, postsynaptic β1 and β3 integrins have been reported to regulate glycine receptor stabilization at the postsynaptic membrane, with the two integrins acting in opposing directions (Charrier et al., 2010).

Finally, the cell adhesion molecule neurofascin has been shown to regulate the formation of a specific subset of inhibitory synapses on the axon initial segment of principal neurons (Ango et al., 2004; Burkarth et al., 2007; Kriebel et al., 2011).

ROLE OF SECRETED FACTORS AND RETROGRADE MESSENGERS AT INHIBITORY SYNAPSES

Above we have described how cell adhesion molecules may provide signals to inhibitory axons from direct cell–cell contacts. However, inhibitory synapses may also be affected by signals from more distal origin. Nearby dendrites or surrounding cells can secrete trophic (or anti-trophic) factors, which may affect inhibitory synapse function and/or formation. Indeed, retrograde signals from the postsynaptic dendrite, such as endocannabinoids, nitric oxide (NO) or brain-derived neurotrophic factor (BDNF), or glutamate spillover from nearby excitatory synapses are known to regulate synaptic release at inhibitory synapses during many forms of short-term and long-term plasticity (Heifets and Castillo, 2009; Regehr et al., 2009; Castillo et al., 2011). Here we discuss secreted factors that have been linked to the formation of inhibitory synapses and that might play a role in activity-dependent regulation of the number of presynaptic terminals made by inhibitory axons.

BRAIN-DERIVED NEUROTROPHIC FACTOR

Brain-derived neurotrophic factor (BDNF) is a secreted neurotrophin that has been shown in many different preparations to promote the formation and maturation of inhibitory synapses by presynaptic modifications (Vicario-Abejón et al., 1998; Huang et al., 1999; Marty et al., 2000; Yamada et al., 2002; Gottmann et al., 2009). Only excitatory neurons produce BDNF (Gottmann et al., 2009; Park and Poo, 2013) and BDNF is released from principal neurons in an activity-dependent manner (Kolarow et al., 2007; Kuczewski et al., 2008; Matsuda et al., 2009), which makes BDNF an attractive candidate molecule to regulate activity-dependent inhibitory synapse formation (Liu et al., 2007). Interestingly, the availability of postsynaptic BDNF signaling in individual neurons was shown to affect the number and strength of inhibitory synapses specifically onto the affected neurons (Ohba et al., 2005; Kohara et al., 2007; Peng et al., 2010). These cell-autonomous effects indicate the potential for BDNF in mediating changes in inhibitory synapses with high synaptic specificity. In excitatory axons, BDNF was shown to reduce the anchoring of synaptic vesicles at presynaptic terminals and thereby increase the exchange of vesicles between boutons (Bamji et al., 2006). It is currently not known if BDNF has a similar effect in inhibitory axons.

NEUREGULIN1

Neuregulin1 is a neurotrophic factor, which exists in various membrane-bound and diffusible isoforms. Mutations (both loss-of-functions and gain-of-function) in neuregulin1 have been linked to schizophrenia (Mei and Xiong, 2008). The main receptor for neuregulin1, ErbB4, is specifically expressed in interneurons (Vullhorst et al., 2009; Fazzari et al., 2010) and is located at postsynaptic densities of excitatory synapses in interneuron dendrites as well as at inhibitory axon terminals. An important role for neuregulin1 is the regulation of excitatory input onto interneurons through postsynaptic ErbB4 (Fazzari et al., 2010; Wen et al., 2010; Ting et al., 2011). Presynaptic ErbB4 can enhance GABA release from inhibitory synapses (Woo et al., 2007; Fazzari et al., 2010) and may affect the number of synapses made by inhibitory axons (delPino et al., 2013). In addition to ErbB4, neuregulin1 isoforms can also activate other receptors resulting in downregulation of postsynaptic $GABA_A$ receptors (Yin et al., 2013). This suggests that neuregulin1 has multiple actions on inhibitory synapses depending on the isoform and activated receptors.

FGF7

Fibroblast growth factors (FGFs) are secreted signaling glycoproteins, which exert their effect by binding to FGF receptor tyrosine kinases (FGFR). In the brain, FGF signaling is important for several developmental processes, including patterning of different brain structures and neurogenesis (Dono, 2003; Reuss and von Bohlen und Halbach, 2003). In addition, FGFs have been implicated as target-derived presynaptic organizers (Umemori et al., 2004). FGF7 is of particular interest, as it localizes specifically to inhibitory synapses in the hippocampal CA3 region, where it is secreted from the postsynaptic membrane and organizes presynaptic release properties (Terauchi et al., 2010). FGF receptors have been shown to directly interact with adenosine A2A receptors (Flajolet et al., 2008), which are important for GABA release (Cunha and Ribeiro, 2000) as well as for GABA uptake from the synaptic cleft (Cristóvão-Ferreira et al., 2009). In this way, FGFR and A2A receptors may act together to regulate GABAergic transmission in the hippocampus.

FACTORS FROM GLIA CELLS

Studies with neuronal and astrocyte co-cultures and astrocyte-conditioned medium have shown that astrocyte-released factors are crucial for synapse formation and plasticity (Elmariah et al., 2005; Christopherson et al., 2005a; Hughes et al., 2010; Crawford et al., 2012). For instance, thrombospondins, oligomeric proteins of the extracellular matrix produced by astrocytes (Christopherson et al., 2005b; Eroglu et al., 2009) are involved in the formation of glutamatergic synapses and the pro-inflammatory cytokine TNFα, coming from glia, (Stellwagen and Malenka, 2006) plays a role in homeostatic plasticity of these synapses. In addition, a different and so far unidentified, protein is secreted by astrocytes, which has been found to increase the density of inhibitory synapses in cultured neurons (Elmariah et al., 2005; Hughes et al., 2010).

GABA

A special secreted factor is the inhibitory neurotransmitter GABA itself. It is well-established that synapse formation does not depend on neurotransmitter release (Verhage, 2000; Harms and Craig, 2005; Schubert et al., 2013). However, the development and maturation of inhibitory synapses are influenced by their neurotransmitter GABA (Li et al., 2005; Huang and Scheiffele, 2008; Huang, 2009; Lau and Murthy, 2012). It was shown that individual axons of parvalbumin-positive basket cells are sensitive to their own GABA release (Chattopadhyaya et al., 2007; Wu et al., 2012) and that the amount of GABA release per vesicle can be regulated by activity (Hartman et al., 2006; Lau and Murthy, 2012). Inhibitory boutons are less dynamic in axons in which GABA release is impaired (Wu et al., 2012) or when GABA receptors are blocked (Schuemann et al., 2013), strongly suggesting that GABA is used as an important activity sensor for regulating activity-dependent presynaptic changes at inhibitory synapses. Both $GABA_A$ and $GABA_B$ receptors have been implicated in mediating this regulation (Fu et al., 2012; Schuemann et al., 2013), but the precise molecular mechanisms remain unknown.

OTHER FACTORS

In addition to cell adhesion molecules and secreted factors, there are many other factors that may affect activity-dependent plasticity of inhibitory axons. For instance, it is well-established that extracellular matrix molecules can play a role in the development and maturation of synapses in the central nervous system and specific interactions between cell adhesion molecules and the extracellular matrix have been revealed (Di Cristo et al., 2007; de Wit et al., 2013; Siddiqui et al., 2013). There are a few studies in which the absence or overexpression of extracellular matrix proteins affected inhibitory synapses specifically (Saghatelyan et al., 2001; Nikonenko et al., 2003; Brenneke et al., 2004; Pavlov et al., 2006; Su et al., 2010), but the underlying mechanisms remain largely unknown.

And finally, while it is clear that presynaptic components are continuously shared and exchanged between inhibitory boutons along the axons, it is not clear how exactly these proteins are dispersed along the axonal shaft. Presumably sharing occurs through passive diffusion of presynaptic proteins through the axonal shaft, but intracellular transport of synaptic cargo could also play a role. Axons contain extensive microtubule and actin networks and there are various motor proteins that deliver and ship transport vesicles, potentially affecting the amount of proteins available for exchange and synapse formation at boutons. For instance, it was shown that intra-axonal movement of mitochondria is enhanced when activity is blocked (Goldstein et al., 2008; Cai and Sheng, 2009; Obashi and Okabe, 2013), but it is not clear if this is due to enhanced motor protein activity or decreased anchoring at synapses. Further research on

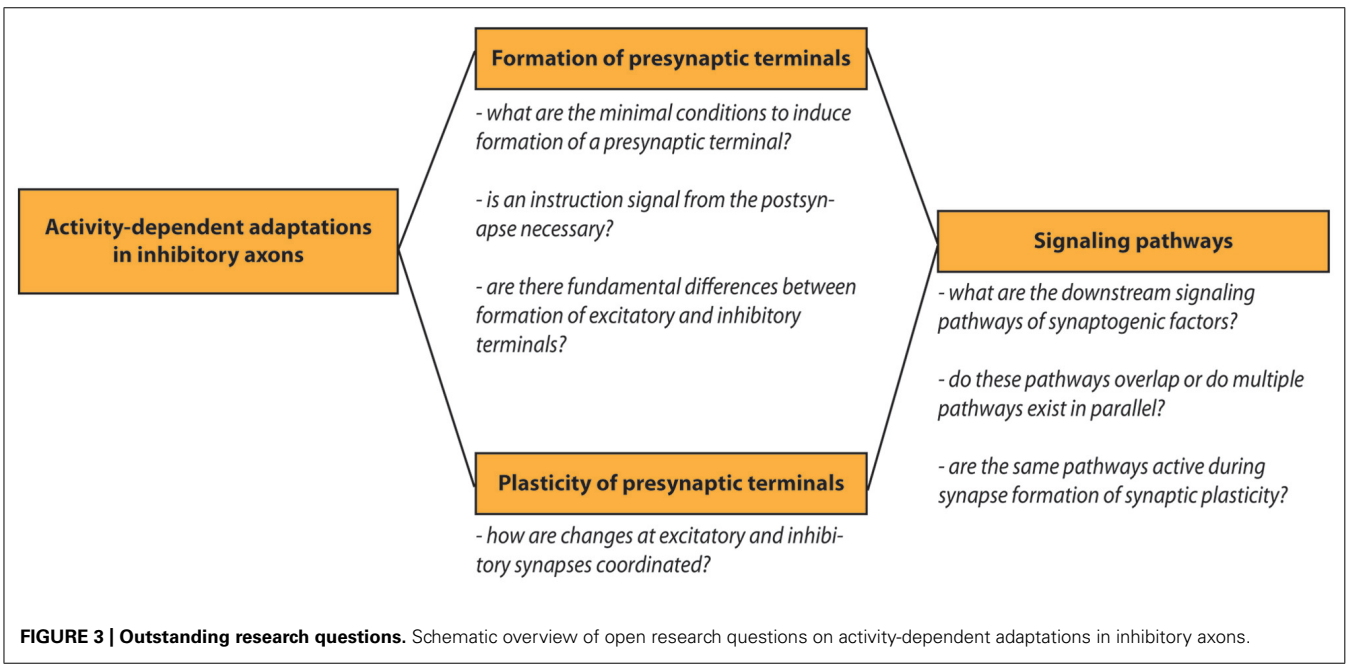

FIGURE 3 | Outstanding research questions. Schematic overview of open research questions on activity-dependent adaptations in inhibitory axons.

the possible activity-dependent regulation of intracellular transport of synaptic cargo (Guillaud et al., 2008; Maas et al., 2009; MacAskill et al., 2009) will be needed to address this issue in the future.

CONCLUSION

Research on activity-dependent adaptations in inhibitory axons continues to generate novel insight in the cellular processes of synapse formation and plasticity. Many open questions remain to be answered in the future and we listed a few of these in a small scheme (**Figure 3**). In this review we have painted a picture of the inhibitory axon as a dynamic structure that can quickly adjust to a changing environment, by responding to local signals from postsynaptic cells via adhesion molecules and to global signals from the local neuronal network. A highly dynamic inhibitory system might serve to quickly respond to changes to allow circuit rearrangements by excitatory connections. For a healthy brain changes at inhibitory and excitatory synapses need to be well-coordinated at all times as subtle defects in this coordination can cause defects in circuitry and may underlie psychiatric disorders. This means that the interplay between plasticity of excitatory and inhibitory synapses is an important factor for the stability of neuronal circuits. The precise response of the inhibitory axon will be determined by the combination of internal and external factors, such as the availability of synaptic proteins within the axon, or the combination of the extracellular factors and cell adhesion molecules that are present at the membrane. It will be an important challenge for future research to unravel the precise molecular and cellular mechanisms and to further uncover pathways that affect synapse formation and plasticity of inhibitory synapses.

ACKNOWLEDGMENTS

The authors like to thank Anne Schuemann, Tara Keck, and Casper Hoogenraad for critical reading of the manuscript and helpful comments. Our research was supported by the People Programme (Marie Curie Actions) of the European Union's Seventh Framework Programme FP7/2007-2013/ under REA grant agreement n 289581 (CPF), and a Marie Curie Reintegration Grant 256284 and Dutch NWO-VIDI grant (CJW).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 27 September 2013; accepted: 30 October 2013; published online: 21 November 2013.

*Citation: Frias CP and Wierenga CJ (2013) Activity-dependent adaptations in inhibitory axons. Front. Cell. Neurosci. **7**:219. doi: 10.3389/fncel.2013.00219*

This article was submitted to the journal Frontiers in Cellular Neuroscience.

frontiers in Cellular Neuroscience

ORIGINAL RESEARCH
published: 09 June 2015
doi: 10.3389/fncel.2015.00220

Network-timing-dependent plasticity

*Vincent Delattre[1], Daniel Keller[2], Matthew Perich[2,3], Henry Markram[1,2] and Eilif B. Muller[2]**

[1] Laboratory of Neural Microcircuitry, Brain and Mind Institute, École Polytechnique Fédérale de Lausanne, Lausanne, Switzerland, [2] Center for Brain Simulation, École Polytechnique Fédérale de Lausanne, Geneva, Switzerland, [3] Department of Biomedical Engineering, Northwestern University, Evanston, IL, USA

Bursts of activity in networks of neurons are thought to convey salient information and drive synaptic plasticity. Here we report that network bursts also exert a profound effect on Spike-Timing-Dependent Plasticity (STDP). In acute slices of juvenile rat somatosensory cortex we paired a network burst, which alone induced long-term depression (LTD), with STDP-induced long-term potentiation (LTP) and LTD. We observed that STDP-induced LTP was either unaffected, blocked or flipped into LTD by the network burst, and that STDP-induced LTD was either saturated or flipped into LTP, depending on the relative timing of the network burst with respect to spike coincidences of the STDP event. We hypothesized that network bursts flip STDP-induced LTP to LTD by depleting resources needed for LTP and therefore developed a resource-dependent STDP learning rule. In a model neural network under the influence of the proposed resource-dependent STDP rule, we found that excitatory synaptic coupling was homeostatically regulated to produce power law distributed burst amplitudes reflecting self-organized criticality, a state that ensures optimal information coding.

Keywords: synaptic plasticity, patch-clamp, acute brain slices, somatosensory cortex, STDP, self-organized criticality, neural networks simulations

OPEN ACCESS

Edited by:
Dion Dickman, University of Southern California, USA

Reviewed by:
Annalisa Scimemi, The State University of New York at Albany, USA
Lisa Mapelli, University of Pavia, Italy

****Correspondence:***
Eilif B. Muller, Center for Brain Simulation, École Polytechnique Fédérale de Lausanne, Biotech Campus, Chemin des Mines 9, CH-1202 Geneva, Switzerland
eilif.mueller@epfl.ch

Received: *17 March 2015*
Accepted: *22 May 2015*
Published: *09 June 2015*

Citation:
Delattre V, Keller D, Perich M, Markram H and Muller EB (2015) Network-timing-dependent plasticity. Front. Cell. Neurosci. 9:220. doi: 10.3389/fncel.2015.00220

Introduction

Periods of synchronous neuronal firing, or bursts of action potentials (APs) in populations of neurons, are ubiquitous in the central nervous system. Bursts can induce long-lasting changes in synaptic efficacy depending on the frequency of bursting, with long-term depression (LTD) being induced by low frequency bursts and long-term potentiation (LTP) being induced at higher frequencies (Bliss and Lomo, 1973; Lynch et al., 1983; Stanton and Sejnowski, 1989; Dudek and Bear, 1992) with notable exceptions (Coesmans et al., 2004). This phenomenon led to the well-known Bienenstock Cooper and Munro (BCM) model of synaptic plasticity (Bienenstock et al., 1982). The relative timing of single spikes generated in connected pairs of neurons can also induce LTP and LTD (Markram et al., 1997; Bi and Poo, 1998), which has led to the well-known Spike-Timing-Dependent Plasticity (STDP) model of synaptic plasticity (Markram et al., 2011). The manner in which these two induction protocols for synaptic plasticity interact is unclear, and it remains to be seen if they can be unified under a common mechanism.

According to the so-called calcium hypothesis, synaptic changes are thought to be determined by the magnitude and time-course of the transient influx of calcium into the synaptic spine induced by pre- and post-synaptic spiking (Bear et al., 1987; Shouval and Kalantzis, 2005; Nevian and Sakmann, 2006; Graupner and Brunel, 2010, 2012). Large calcium influxes are thought to induce potentiation, whereas moderate and prolonged calcium influxes are thought to induce depression (Bienenstock et al., 1982; Nevian and Sakmann, 2006). A network burst induced transient

reduction in extracellular calcium would reduce the magnitude of calcium influx into the spine approximately proportionally (Egelman and Montague, 1999; Wiest et al., 2000), and could subsequently alter the outcome of plasticity. In particular, spiking motifs yielding LTP could instead yield LTD when embedded in a network burst.

This shifting of the direction of plasticity in bursting networks toward LTD is an interesting observation, as it is a possible mechanism for counteracting run-away potentiation in networks of neurons with on-going synaptic plasticity. Increases in synaptic coupling between excitatory neurons are known to induce a transition to bursting activity regimes, as has been reported in previous theoretical studies (Tsodyks et al., 2000; Kudela et al., 2003) and under pathological experimental conditions where synaptic up-scaling was induced by activity deprivation (Trasande and Ramirez, 2007). While homeostatic mechanisms have been proposed to down-regulate synaptic strengths if neuronal firing rates become excessive (Turrigiano et al., 1998; Trasande and Ramirez, 2007), such mechanisms have been shown to be insufficient to maintain network stability in simulations of networks of neurons incorporating empirically constrained STDP models at excitatory synapses (Zenke et al., 2013). One important reason for this is that such mechanisms are insensitive to the transition to the network bursting state, which occurs with only minor changes in neuronal firing rates. The proposed interplay between network bursting activity and STDP could provide negative feedback allowing fine homeostatic control to be maintained in the presence of on-going synaptic plasticity, and thus to maintain states of criticality observed in cortical networks (Beggs and Plenz, 2003; Priesemann et al., 2009, 2013).

To gain insight into the proposed interaction of STDP and network bursting activity, we investigated *in vitro* the effect of precisely timed network bursts on STDP at excitatory synaptic inputs to layer 5 pyramidal neurons where the STDP phenomenon was first reported (Markram et al., 1997). STDP protocols known to induce LTP and LTD were applied, and network bursts were induced at precise timings before, during, or after the STDP pairing protocols using the electrodes of a multi-electrode array (MEA) located in layer 5. The pairing of STDP events with network bursts can influence the plasticity outcome by altering the timing relationship in the pre–post spike motif due to the additional spikes, and by changes in context due to the network burst (such as voltage, competition for resources, etc.). To separate the former effects from the latter, we performed burst-spike-substitution (BSS) experiments whereby the MEA burst was replaced with an excitatory postsynaptic potential (EPSP) paired with a simultaneous post-synaptic AP.

Our main experimental finding is that certain specific timings of network bursts relative to the STDP events can induce flips of LTD into LTP and LTP into LTD, which cannot be accounted for by the BSS protocols, and thus on pre–post spiking alone. We propose that the observed flips are manifestations of positive and negative synaptic cooperativity, respectively, for which a number of mechanisms have been proposed. We hypothesize that *negative cooperativity* could be due to the depletion of critical resources needed for LTP, perhaps through the depletion of an intracellular messenger (Fonseca et al., 2004), or the transient reduction of extracellular calcium at synaptic junctions immediately following network bursting (Egelman and Montague, 1999; Wiest et al., 2000).

We further hypothesize that the observed negative cooperativity could have an important role in the maintenance of the excitation–inhibition balance and of network criticality in the presence of on-going synaptic plasticity. To evaluate this hypothesis, we employ simulations of networks of neurons incorporating an empirically constrained STDP rule (Morrison et al., 2007), and augment it with a resource depletion term implementing a shift of STDP outcomes from LTP to LTD when embedded in a network burst. Networks including the resource depletion term are found to induce a transition to a state of criticality in the network (Beggs and Plenz, 2003; Priesemann et al., 2009, 2013). The proposed resource-dependent interaction between network activity and STDP therefore represents a novel mechanism for the homeostatic regulation of the network activity regime.

Materials and Methods

Electrophysiology

In accordance with the Swiss national and institutional guidelines, 300 μm thick sagittal brain slices were prepared from somatosensory cortex of postnatal days 13–17 Wistar rats of either sex in iced artificial cerebrospinal fluid (ACSF) containing (in mM) 125 NaCl, 2.5 KCl, 25 D-glucose, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 2 $CaCl_2$, and 1 $MgCl_2$; all chemicals from Sigma–Aldrich (St. Louis, MO, USA or Merck, Darmstadt, Germany), using a HR2 vibratome (Sigmann Elektronik, Heidelberg, Germany). The primary somatosensory cortex was manually dissected and isolated to obtain rectangular slices of 5–7 mm width and containing the neocortex in its entire height. Optimal slices, with apical cell dendrites running parallel to the slice surface, were selected for recordings. Slices were incubated at 22°C for 30–60 min until mounting in the recording chamber. Slices were mounted on a 3D-MEA with 60 pyramidal platinum electrodes (electrode basis: 40 μm × 40 μm, electrode height: 50–70 μm, electrode interspacing: 200 μm; Qwane Bioscience SA, Lausanne, Switzerland) after evaporation of a mounting solution of 0.14 mg/L nitrocellulose in an ethanol (99%) – methanol (1%) mixture. Cells were visualized by infrared differential interference contrast video microscopy using a camera (VX 55, Till Photonics, Gräfelfing, Germany) mounted on an upright microscope (BX 51WI, Olympus, FI, Japan) fitted with a 40× objective (LUMPLAN, Olympus). Whole-cell recordings were performed using Axopatch 200B amplifiers (Molecular Devices, Union City, CA, USA). Data acquisition, sampled at 5–10 kHz, was performed via an ITC-18 board (Instrutech Co, Port Washington, NY, USA), connected to a computer running IgorPro (Wavemetrics, Portland, OR, USA). The voltage signal was filtered with a 2 kHz Bessel filter. Multiple somatic whole cell recordings (1–3 cells simultaneously) were performed using patch pipettes pulled with a P-97 Flaming/Brown micropipette puller (Sutter Instruments Co, Novato, CA, USA) with an initial

resistance of 8–10 M Ω. Patch pipettes were filled with standard intracellular solution (ICS) containing (in mM): 110 K-gluconate, 10 KCl, 4 ATP-Mg, 10 phosphocreatine, 0.3 GTP, 10 *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (pH 7.3), and 0.5% biocytin. Recordings were not corrected for the liquid junction potential between ACSF and ICS (–14 mV). Variation in the cell input resistance was measured from beginning to end of the experiment, and all cells having a change in input resistance greater than 33% were excluded. Cell access resistance was typically less than 20 MΩ.

Electrical Stimulation

A STDP protocol known to induce LTP (the STDP$^+$ event) was applied as a 50 Hz train of three APs with a single evoked EPSP 10 ms earlier (Nevian and Sakmann, 2006; **Figure 1B**). A STDP protocol known to induce LTD (the STDP$^-$ event) was applied as a 50 Hz train of three APs with a single evoked EPSP 10 ms later (Nevian and Sakmann, 2006; **Figure 1C**). EPSPs were evoked by extracellular stimulation using an extracellular pipette located near the basal dendrites of the patched-cell, and paired with APs evoked with supra-threshold intracellular current injection, as previously described (Nevian and Sakmann, 2006). Pairings were repeated 60 times at a frequency of 0.1 Hz. EPSPs were monitored at a frequency of 0.1 Hz, for 10 min prior to pairing to record the baseline and for more than 1 h post pairing. Network bursts were evoked by extra-cellular electrical stimulation of layer 5 (STG2008 stimulator, Multi Channel System, Reutlingen, Germany) using a 3D-MEA (electrode basis: 40 μm × 40 μm, electrode height: 50–70 μm, inter-electrode spacing: 200 μm; Qwane Bioscience SA, Lausanne, Switzerland). Stimulation strength was tuned to trigger a single AP in every patched cell (1–2 V biphasic pulses; 1 ms duration in each polarity). On average, the network burst failed to evoke a spike in 6.8% of the cases, triggered a single spike in 89.2% of the cases, and two spikes in 4% of the cases (Supplementary Figure S1). We never observed a network burst causing more than two spikes in the patched cells. The latency of the first evoked spike was 3.7 ± 0.2 ms (range 1.4–10.8 ms, $n = 3960$ network bursts recorded in 66 cells). Due to the symmetry of the MEA evoked network burst, pre-synaptic spiking in excitatory cells during the burst is assumed to mirror post-synaptic spiking. The time interval ΔT between EPSP and the network burst was defined as the time between EPSP digital trigger and the network burst digital trigger.

Bursts were evoked before (–20 ms), simultaneous to (0 ms) or at the end of (50 ms) an STDP$^+$ event, and at the beginning of (–50 ms), simultaneous to (0 ms), or after (20 ms) an STDP$^-$ event. The timing of the burst with respect to the STDP$^-$ protocol to was chosen to exactly mirror all tested protocols for LTP. The combined burst-STDP event pairing was applied at a frequency of 0.1 Hz.

Burst-spike-substitution experiments replaced the network burst with an EPSP paired with a simultaneous post-synaptic AP for burst-STDP pairings, to mirror the pre- and post-synaptic spiking as seen by a synapse during a burst-STDP event (assuming the predominant case above that bursts trigger a single pre- and post-synaptic spike), but without the network context.

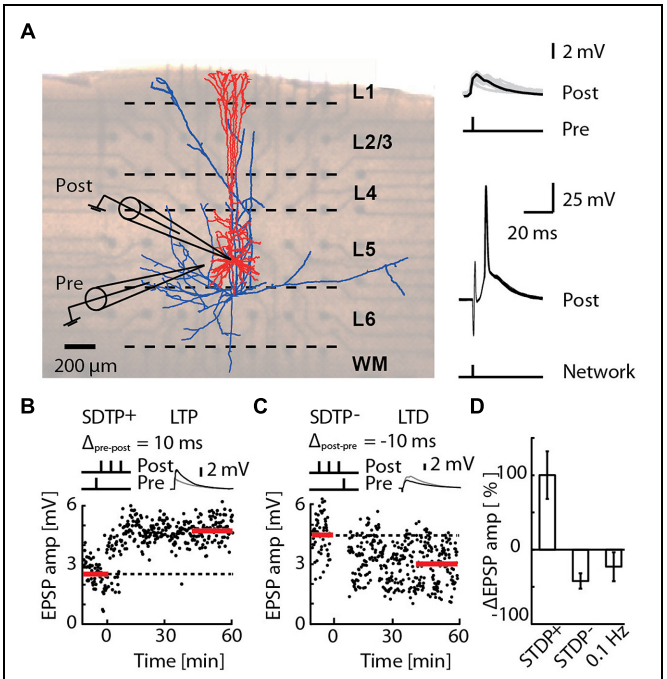

FIGURE 1 | Induction of long-term potentiation (LTP) and long-term depression (LTD) by pairing an EPSP with a short burst of action potentials (APs), or by network bursting. (A) Cortical slice mounted on a 3D-multi-electrode array (MEA), with a reconstruction of a layer 5 pyramidal neuron (blue axons, red dendrites) overlaid (left). A whole-cell patch of the pyramidal neuron (post) receiving an EPSP (pre) evoked by extracellular electrical stimulation (upper-right). The post-synaptic responses due to network bursts evoked by MEA stimulation in the region of layer 5 are overlaid for 30 repetitions (lower-right). **(B)** A typical recording for the STDP$^+$ paradigm (STDP$^+$; black circles). EPSP amplitude was measured every 10 s (baseline and final amplitude indicated by the red line). **(C)** A typical recording for the STDP$^-$ paradigm (STDP$^-$; black circles). **(D)** Mean change in EPSP amplitude for STDP$^+$ (ΔEPSP amp. = 103 ± 33%, $n = 11$), STDP$^-$ (ΔEPSP amp. = −44 ± 10%, $n = 6$) and 0.1 Hz network bursting (ΔEPSP amp. = −25 ± 20%; $n = 9$).

Experimental Data Analysis and Statistics

Experimental data analysis was performed in Matlab (The MathWorks, Inc., Natick, MA, USA) with custom scripts. EPSP amplitude was monitored for an hour and 20 min. Baseline EPSP was acquired over the first 10 min, followed by 10 min of pairing, as described above. The final EPSP amplitude was averaged over the last 20 min of recordings. EPSP failure or EPSPs that caused the cell to spike were excluded from the analysis. However, if after the pairing, a cell fired 100% of the time following the EPSP onset within an averaging time window (20 repetitions), we assumed a strong potentiation to have occurred and set the synaptic gain to a value of 5 for this time period. Data are presented as the mean ± SEM. Paired Student's *t*-tests were applied as statistical tests, and statistical significance was asserted for: $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

Network Simulations

We simulated a network of 1000 integrate-and-fire (IF) neurons (of which 80% are excitatory and 20% inhibitory)

arranged on a 10 × 10 × 10 lattice, corresponding to a 200 μm × 200 μm × 200 μm volume of cortex in an active state. Neuron parameters were fit to publicly available Hodgkin–Huxley type neuron models (Destexhe et al., 1998) for f-vs-I curves and noise current injections. Excitatory neurons contained a spike triggered conductance inducing spike-frequency adaptation (Muller et al., 2007). Each neuron had 1000 excitatory (AMPA) and 250 inhibitory ($GABA_A$) conductance-based synapses with a peak conductance of 2 nS (except plastic synapses) and time constants of 1.5 ms and 10 ms, respectively. Consistent with anatomy, 10–20% of the synaptic inputs originated from neurons inside the network (140 exc. → exc., 200 exc. → inh., 50 inh. → exc., 50 inh. → inh.), and delays were computed as $d\beta\,(1+\xi)$, where d is the distance, $\beta = 0.25$ ms/unit lattice and ξ is a random number drawn from an exponential distribution with mean of 0.2. Extrinsic input was modeled by Poisson processes with firing rates parameterized separately for excitatory and inhibitory input, 6 and 10.5 Hz, respectively. These rates were determined numerically to be consistent with excitatory and inhibitory model neuron firing rates resulting from application of exclusively Poissonian input at these rates at all synapses. Neuronal properties in the network are consistent with the "high-conductance state" (Destexhe et al., 2003). We used the Power-law STDP rule (Morrison et al., 2007) parameterized for cortical (Froemke and Dan, 2002) conductance-based synapses to achieve a mean of ∼1.9 nS under extrinsic input alone as follows: $\tau_+ = 14$ ms; $\tau_- = 34$ ms; $w_0 = 4.29 \times 10^{-2}$ nS; $\mu = 0.4$; $\lambda = 0.1$; and $\alpha = 4.8 \times 10^{-2}$. For the computation of STDP time differences, connection transmission delays were treated as half axonal and half dendritic. Where stated, the STDP rule was augmented with a model for activity-dependent resource availability as described in the main text. The network and neuron models were implemented using the PyNN modeling language (Davison et al., 2009) with the NEURON simulator backend (Hines and Carnevale, 1997) and are publicly available at: https://neuralensemble.org/svn/PyNN/trunk/examples/iaf_sfa_relref/

Resource Dependent STDP

To implement the hypothesized effects of resource depletion, such as extracellular calcium, on STDP as a mechanism to flip LTP into LTD, we added a resource depletion term to a standard STDP learning rule (Morrison et al., 2007) in the network model. We modeled resource depletion caused by network activity by assuming the equilibrated resource availability for any fixed average network firing rate, α, has the form $\eta_0(\alpha) = \left(1+\alpha/k\right)^{-1}$, where k is the depletion rate constant (k was assigned to 20 Hz to allow for a 50% resource depletion during sustained 20 Hz network activity). The dynamic resource availability $\eta(t)$ was then computed by low pass-filtering $\eta_0(\alpha\,(t))$ as follows:

$$\frac{d}{dt}\eta(t) = \frac{\eta_0(\alpha\,(t)) - \eta(t)}{\eta_0(\alpha\,(t)) \cdot \tau_\eta},$$

where $\alpha(t)$ is a continuous estimator of the average networking firing rate (low-pass filtered network spiking with a filter time constant $\tau_\alpha = 2.5$ ms and normalized by network size), τ_η is the recovery time constant of the resource availability (assumed to be 100 ms) in the absence of network activity, and the factor of $\eta_0(\alpha\,(t))$ in the denominator ensures that depletion is fast while recovery is slow. Biologically, the hypothesized resource depletion is likely to be a local phenomenon, but the extent of the locality remains unknown. As further experiments reveal the actual distance over which the network could act on a synapse, sub-volumes can be defined and $\eta(t)$ computed for each voxel in the context of the whole network. We, however, did not define sub-volumes of the network and considered the firing of all neurons when computing $\eta(t)$, representing the average resource availability for the entire network volume.

To regulate LTP induced by $STDP^+$ events, we scaled the computed synaptic weight change ΔW_+ by the resource availability $\eta(t)$ and implemented the scaling as

$$\Delta W_+^{'} = \gamma\,(\eta\,(t)) \cdot \Delta W_+\,,$$

where $\gamma(\eta)$ is a sigmoidal resource modulation function,

$$\gamma(\eta\,(t)) = \frac{2}{1+\exp\left(\frac{\eta^* - \eta(t)}{m}\right)} - 1,$$

with $\eta^* = 0.6$ defining the LTP–LTD reversal point and $m = 0.03$ the steepness of the reversal. In this model, synapses active at the onset of bursts consume resources needed to potentiate, thus forcing later activated synapses to depress.

Criticality Analysis

We fit a power-law using methods described in Clauset et al. (2009) to the cumulative burst size distribution of the network PSTH (dt = 2 ms) normalized by the SD of the activity of the sub-threshold network model ($\omega = 1.2$ nS) without plasticity. The threshold for burst detection was set to the mean network firing rate normalized by the SD of the network firing rate when $\omega = 1.2$ nS plus two. The branching parameter (σ) was computed as previously described (Beggs and Plenz, 2003; Priesemann et al., 2009).

Results

Network-Timing-Dependent Plasticity

We investigated in acute slices of juvenile rats mounted on a 3D-MEA (**Figure 1A**) the effect of precisely timed network bursts on STDP at excitatory synaptic inputs to layer 5 pyramidal neurons. STDP protocols known to induce LTP (the $STDP^+$ event) and LTD (the $STDP^-$ event) were applied as a 50 Hz train of three APs with, respectively, a single evoked EPSP 10 ms earlier or later (Nevian and Sakmann, 2006; **Figure 1**, see Materials and Methods). The $STDP^+$ event reliably induced LTP (**Figures 1B,D**; ΔEPSP = 103 ± 33%, $n = 11$) and the $STDP^-$ event reliably induced LTD (**Figures 1C,D**; ΔEPSP = –44 ± 10%, $n = 6$), also as previously reported (Nevian and

Sakmann, 2006). Network bursts were evoked by extra-cellular electrical stimulation of layer 5 using a 3D-MEA (see Materials and Methods). On average, the network burst failed to evoke a spike in 6.8% of cases, triggered a single spike in 89.2% of cases, and two spikes in 4% of cases (Supplementary Figure S1). We never observed a network burst causing more than two spikes in the patched cells. The latency of the first evoked spike was 3.7 ± 0.2 ms (range 1.4–10.8 ms, $n = 3960$ network bursts recorded in 66 cells). Bursts alone evoked at 0.1 Hz induced LTD in seven out of nine cells (ΔEPSP = –41 ± 12%, $p <$ 4e-3, one sample t-test to baseline 0%), whereas one cell exhibited no significant change (ΔEPSP = 19 ± 20%), and one cell expressed LTP (ΔEPSP = 111 ± 15%). Pooling all these cells in a single group led to a relatively high variability, and a skewed distribution (**Figure 1D**; ΔEPSP = –25 ± 20%; $n = 9$). Taken together, these results indicate that bursts alone evoked at 0.1 Hz generally induced LTD, consistent with previous reports for 1 Hz evocation of population EPSPs in hippocampal slices (Stanton and Sejnowski, 1989; Dudek and Bear, 1992), however, a small sub-population of cells expressing LTP cannot be excluded.

We then examined the interaction between burst-induced LTD and STDP$^+$ events (**Figures 2A–C**). The LTP induced by STDP$^+$ remained unchanged when bursts coincided with the STDP$^+$ events ($\Delta T = 0$; ΔEPSP = 109 ± 64%, $p = 0.17$; $n = 8$), indicating that plasticity driven by relative spike timing in a specific synaptic pathway is unaffected by simultaneous network bursts. This result also indicates that LTD normally induced by such network bursts is blocked by simultaneous STDP$^+$ events. However, when bursts preceded the STDP$^+$ event by 20 ms, LTP flipped into LTD with a magnitude comparable to that of the burst-induced LTD (ΔT = –20 ms; ΔEPSP = –21 ± 7%, $p = 3e^{-4}$, $n = 7$), indicating that immediately preceding network bursts block STDP$^+$-induced LTP. Conversely, STDP$^+$ events fail to block LTD induced by preceding bursts. Burst- and STDP$^+$-induced plasticity mutually canceled when bursts were evoked 5s before or after STDP$^+$ events (ΔT = ±5000 ms; ΔEPSP = 7 ± 24%, $p = 2.8e^{-2}$; $n = 8$) or 50 ms after the STDP$^+$ event (ΔT = +50 ms; ΔEPSP = 8 ± 21%, $p = 9.3e^{-3}$; $n = 14$). All statistical tests comparing each protocol to baseline and to other protocols are provided in Supplementary Table S1.

We next tested for interactions between burst-induced LTD and STDP$^-$ events (**Figures 2D–F**). Bursts that were simultaneous, 50 ms before and 5s before or after STDP$^-$ events had no cumulative effect on the LTD induced by the STDP$^-$ events, indicating that LTD is saturated. This saturation suggests that burst- and STDP$^-$-induced LTD share expression mechanisms. Surprisingly, LTD flipped into LTP when bursts immediately followed STDP$^-$ events ($\Delta T = 20$ ms, 81 ± 50%, $p = 3.5e^{-2}$, $n = 9$), indicating that the combination of LTD expression mechanisms induced by both burst and STDP$^-$ events results in the expression of LTP.

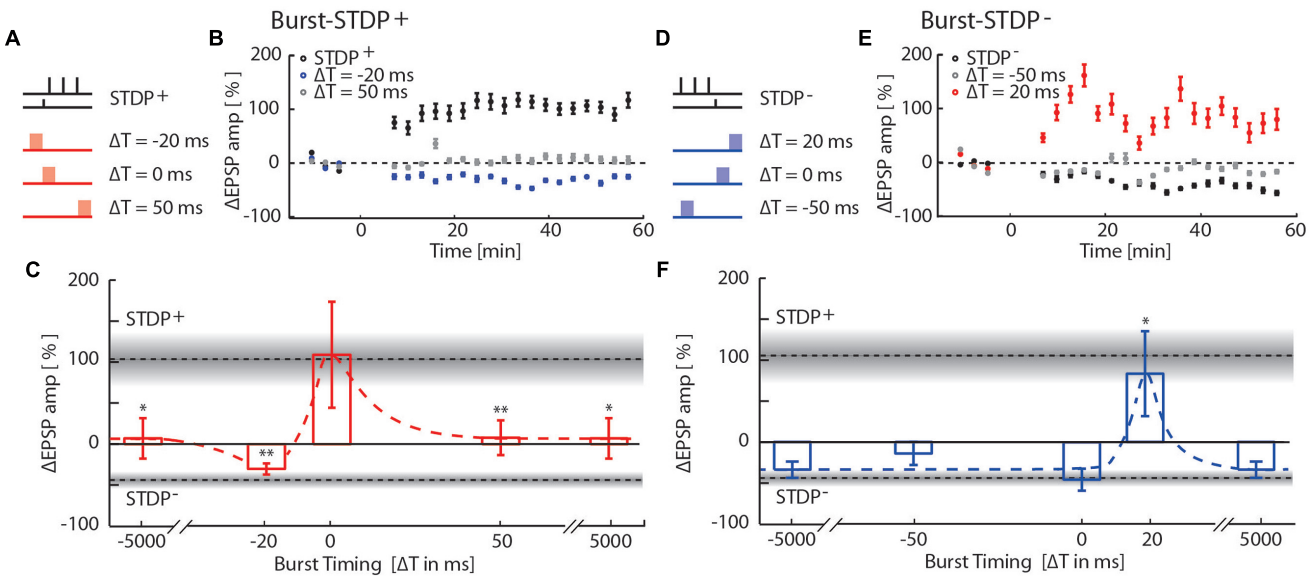

FIGURE 2 | Network-timing-dependent modulation of Spike-Timing-Dependent Plasticity (STDP). (A) Pairing of STDP$^+$ with MEA evoked network bursts at various relative timings with respect to the presynaptic STDP$^+$ input. **(B)** EPSP amplitude changes due to burst-STDP$^+$ pairings when the burst precedes (ΔT = –20 ms; blue circles) or follows (ΔT = 50 ms; gray circles) the STDP$^+$ event (black circles). **(C)** Summary of changes in EPSP amplitude for the various STDP$^+$ protocols. Dotted lines and gray shaded areas show the mean ± SEM EPSP amplitude change induced by STDP$^+$ and STDP$^-$. Depending on its relative timing, the burst either flipped LTP to LTD (burst preceding; ΔT = –20 ms), blocked LTP (burst following; ΔT = 50 ms, ± 5 s), or had no effect on the STDP pairing (simultaneous burst; ΔT = 0 ms). **(D)** Pairing of STDP$^-$ with network bursts at various timings. **(E)** EPSP amplitude changes due to burst-STDP$^-$ pairings when the burst precedes (ΔT = –50 ms; gray circles) or follows (ΔT = 20 ms; red circles) the STDP$^-$ event (black circles). **(F)** Summary of changes in EPSP amplitude for the various STDP$^-$ protocols. Dotted lines and gray shaded areas show the mean ± SEM EPSP amplitude change induced by STDP$^+$ and STDP$^-$. STDP$^-$ induced LTD is unaffected unless the burst shortly follows the STDP$^-$ event (ΔT = 20 ms).

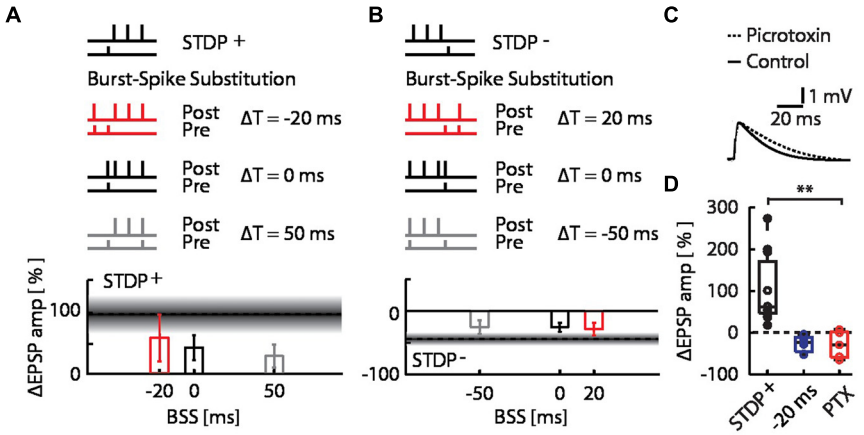

FIGURE 3 | Burst-spike-substitution (BSS) protocols do not explain the flip from LTP to LTD and LTD to LTP, and inhibitory circuits do not contribute to the flip from LTP to LTD. (A) EPSP amplitude changes for BSS protocols of burst-STDP$^+$ pairings, with simultaneous AP and EPSP at $\Delta T = -20$ ms (red), $\Delta T = 0$ ms (black) and $\Delta T = 50$ ms (gray). All timings yielded LTP. **(B)** EPSP amplitude changes for BSS protocols of burst-STDP$^-$ pairings, with simultaneous AP and EPSP at $\Delta T = 20$ ms (red), $\Delta T = 0$ ms (black) and $\Delta T = -50$ ms (gray). All timings yielded LTD. **(C)** Average normalized EPSP baseline waveforms for control cells, and cells with intracellular picrotoxin (PTX) reveal the effect of PTX on the evoked response by the stimulation with the extracellular pipette. **(D)** EPSP amplitude change for the STDP$^+$ event (black) with the network burst at $\Delta T = -20$ ms (blue), and with the network burst at $\Delta T = -20$ ms with PTX (red).

The pairing of STDP events with network bursts can influence the plasticity outcome by alterations of the timing relationship in the pre–post spike motif due to the additional spikes, and by changes in context due to the network burst. To determine whether the observed interaction between bursts and STDP events can be explained entirely by the single pre-synaptic and single post-synaptic spikes added to the STDP pairing protocol by the MEA stimulation, we repeated the burst-STDP pairing experiments substituting the burst with a single EPSP simultaneous to an AP (**Figures 3A,B**; BSS, see Materials and Methods). In terms of pre- and post-synaptic spiking, this BSS is equivalent to MEA stimulation (see Materials and Methods and Supplementary Figure S1). BSS could not account for the flip of LTP into LTD due to a burst 20 ms before the STDP$^+$ event (**Figure 3A**; red bar), nor the flip of LTD into LTP due to a burst 20 ms after the STDP$^-$ event (**Figure 3B**, red bar). All other BSS timings yielded changes in EPSP amplitudes that were consistent with their respective burst-STDP pairings (**Figures 3A,B**). These data imply that multiple inputs to the neuron from the bursting network are required to induce the observed flips in directionality of plasticity.

Finally, we examined whether inhibitory synaptic inputs activated by the burst played a role in the observed flip of LTP into LTD. We repeated the burst-STDP$^+$ experiment with the burst 20 ms before the STDP$^+$ event, while blocking inhibitory currents in the patched cells with intracellular picrotoxin (PTX; **Figure 3C**; Paille et al., 2013). We found that the bursts still flipped the LTP into LTD (**Figure 3D**; burst at $\Delta T = -20$ ms + PTX; open red circles; ΔEPSP amp $= -26 \pm 14\%$, $p = 0.18$ against STDP$^+$ and $p = 0.38$ against burst + STDP$^+$, $n = 5$), indicating that inhibitory inputs do not play a significant role in burst-dependent STDP.

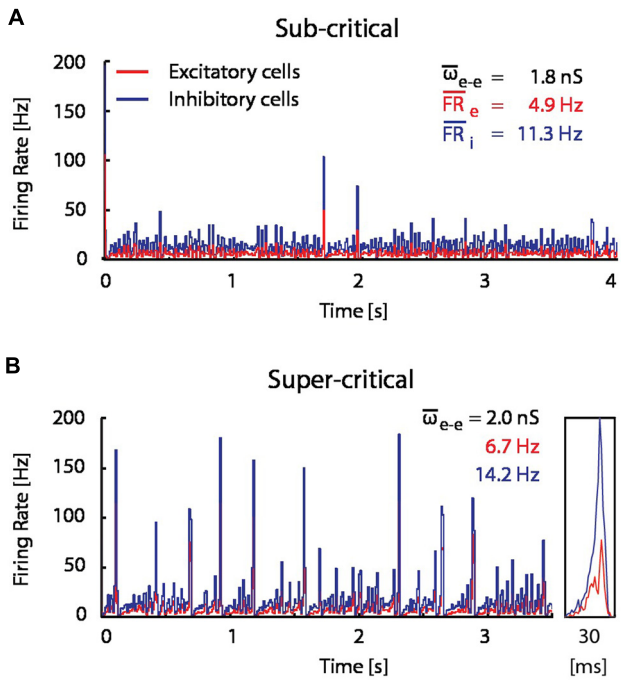

FIGURE 4 | A modest increase in excitatory coupling leads to spontaneous network bursting. Firing rate of recurrent randomly connected network of 1000 integrate-and-fire (IF) neurons with 20% inhibitory cells in an active state (see Materials and Methods); $\bar{\omega}_{e-e}$ is the mean excitatory coupling, $\overline{FR_e}$ and $\overline{FR_i}$ are the mean firing rate of excitatory (red) and inhibitory (blue) cells in the network, respectively. The simulated network changes its state from sub-critical **(A)**, to super-critical (spontaneous periodic bursting, **B**) after a 10% increase of mean synaptic weight for excitatory–excitatory connections. (Inset) Example of a typical network burst is shown to the right.

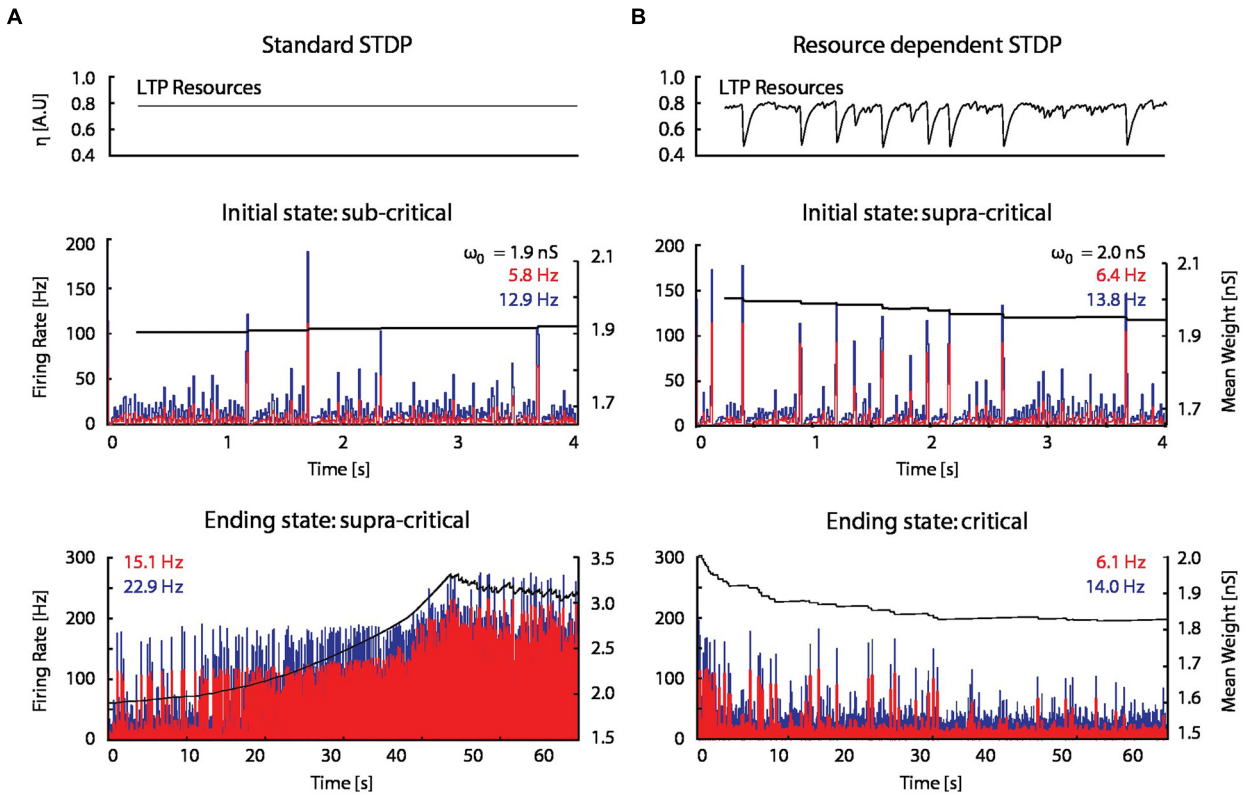

FIGURE 5 | A STDP model with activity-dependent resource consumption counter-balances runaway potentiation. (A) With a standard STDP rule, the firing rate and mean synaptic weight of a network initialized in a sub-critical state drift toward non-physiological values in a supra-critical state; ω_0 is the initial mean excitatory–excitatory synaptic strength. **(B)** The resource-dependent STDP rule drives networks initialized in a super-critical state toward a critical state.

Taken together, these data suggest that the observed flips of LTD into LTP and LTP into LTD could be manifestations of positive and negative synaptic cooperativity, respectively. We hypothesized that the flip from LTP into LTD (negative cooperativity) could be due to the depletion of critical resources needed for LTP.

Resource-Dependent Regulation of STDP

In order to assess the significance of the observed flip from LTP into LTD, and the hypothesized resource depletion on STDP, we proposed a resource-dependent STDP learning rule (see Materials and Methods), and examined its implications in network simulations. We simulated the network dynamics and evolution of synaptic weight distributions without STDP, with STDP and with resource-dependent STDP in a simplified network model consisting of 1000 IF neurons (80% excitatory, 20% inhibitory; see Materials and Methods). Without STDP, network dynamics are highly sensitive to the mean excitatory–excitatory synaptic coupling. For example, a mere 10% increase in coupling is sufficient to drive the network from a sub-critical regime exhibiting aperiodic occurrence of spontaneous bursts at low frequencies (**Figure 4A**), to a supra-critical regime exhibiting frequent spontaneous and periodic network bursts (**Figure 4B**; Beggs and Plenz, 2003; Shew and Plenz, 2013). This transition from sub- to supra-critical activity regimes has been reported in previous theoretical studies (Tsodyks et al., 2000; Kudela et al., 2003) and under pathological experimental conditions where synaptic up-scaling was induced by activity deprivation (Trasande and Ramirez, 2007).

When STDP was introduced into the model network (see Materials and Methods), spontaneous network bursts resulted in more LTP than LTD on average, which gradually increased excitatory–excitatory coupling, and in turn led to an increase in burst frequency and amplitude. This positive feedback drove synaptic weights and network activity to non-physiological regimes (**Figure 5A**).

When the proposed resource-dependent STDP learning rule was introduced into the model, we found that when networks were initialized with strong excitatory–excitatory coupling that caused supra-critical activity and spontaneous network bursting at low rates, the network converged to a critical level (**Figure 5B**) in which regular periodic network bursts were replaced with low frequency irregular bursts. Mean synaptic weights also decreased and stabilized at an intermediate value (**Figure 5B**; bottom panel), consistent with experimental observations (**Figure 6A**; Bear et al., 1987). On the other hand, the mean synaptic weight for sub-critical networks was found to increase toward

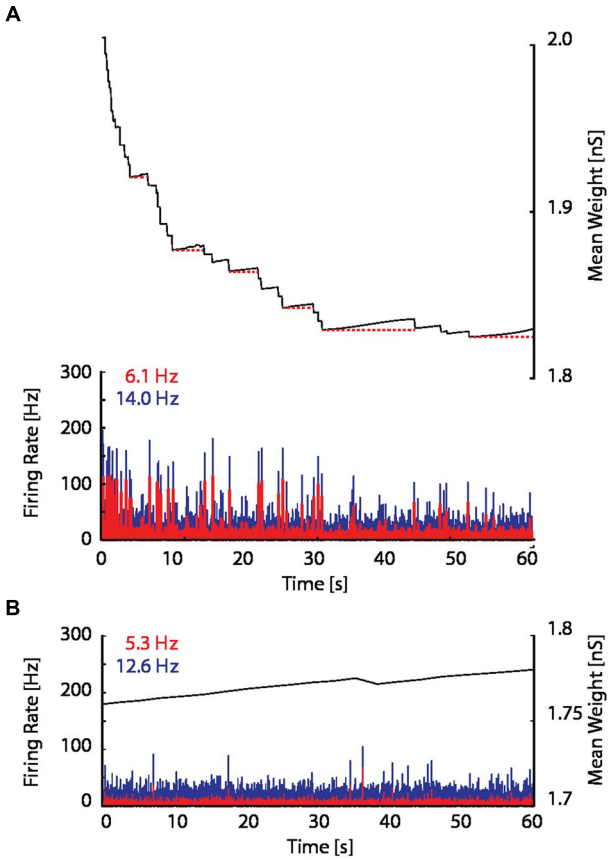

FIGURE 6 | Excitatory coupling is rising due to on-going synaptic activity. (A) The mean synaptic weight (black), as in the bottom panel in **Figure 5B**, but with an enlarged scale. Red dotted horizontal lines have been drawn to indicate the progressive increase of the mean synaptic weight between network bursts. **(B)** The firing rate and mean synaptic weight of a network with the resource-dependent STDP rule when the network is initialized in a sub-critical state. The mean synaptic weight is continuously rising, and ultimately will reach the threshold for network bursting.

the same intermediate value (**Figure 6B**). Resource-dependent STDP therefore homeostatically regulates synaptic weights to maintain a mean value just below the threshold for synchronous bursting.

Self-Organized Criticality Emerges from Resource-Dependent STDP

To determine whether the proposed resource-dependent STDP gives rise to what is known for physical systems as a self-organized critical state (Bak et al., 1988), we analyzed the bursting statistics under the different network conditions described above. Self-organized criticality is indicated when the cumulative probability distribution of event amplitudes follows a power law (Beggs and Plenz, 2003). We therefore plotted the cumulative probability of a burst of a given event size occurring for the network dynamics without STDP, with STDP, and with resource-dependent STDP (**Figure 7A**). Without STDP and at the implemented mean excitatory–excitatory synaptic couplings (ω = 1.2, 2, and 3 nS), the burst-size distribution did not follow a power law distribution (**Figure 7A**; gray to black dotted lines). With STDP alone, the distribution was markedly different from a power law (**Figure 7A**; red dotted line). With resource-dependent STDP, all bursts up to 15 times the amplitude of the minimally detected burst followed a power law. The distribution began to deviate when the bursts engaged more than about 20% of the neurons in the network (largest burst involves around 45% of the neurons), equivalent to 14 times the mean network activity (**Figure 7A**; blue dotted line).

Another measure of criticality is the branching parameter (see Materials and Methods), which also gives an indication of the efficiency of the network state to convey information (Beggs and Plenz, 2003; Shew and Plenz, 2013). Networks without STDP have a fixed branching parameter (**Figure 7B**; gray lines). Networks with STDP alone transitioned to a supra-critical state with a branching parameter greater than 1 (**Figure 7B**; red line), and were equivalent to networks without STDP and strong excitatory–excitatory synaptic coupling (**Figure 7B**; solid black line). With resource-dependent STDP, networks converged to a state with a branching parameter around 1, indicating a critical state (**Figure 7B**; blue line).

Discussion

Spike-Timing-Dependent Plasticity provides a mechanism to modify the synaptic weight of inputs to a neuron according to their relative timing with respect to the back-propagating AP. We report here a phenomenon we refer to as *network-timing-dependent plasticity* (NTDP), whereby local spike-timing-dependent plasticity of individual synaptic pathways is regulated by the relative timing of synchronous bursts generated by the network. NTDP can regulate STDP by blocking (acting in the opposite plasticity direction), saturating (acting in the same direction) and flipping (acting in the same or opposite direction and crossing a threshold of interaction) depending on the relative timing of synchronous network activity. Positive cooperativity (flipping LTD into LTP) could be explained by cooperative interactions between weak and strong inputs (Levy and Steward, 1983; Sjöström et al., 2001), multiple input-driven facilitation of the bAP (Sjöström and Häusser, 2006) or by the *threshold accumulation* of resources (same directions of plasticity), such as intracellular calcium levels (Lisman, 1989; Shouval et al., 2002; Graupner and Brunel, 2010, 2012). In the case of the latter, calcium influxes of individual events would not cross the threshold concentration for LTP and consequently lead to depression, but together more easily cross a threshold concentration for LTP induction (Lisman, 1989). Such a hypothesized positive cooperativity would, however, require a temporal separation to explain why LTD was not flipped into LTP when the burst- and $STDP^-$-induced LTD events occurred simultaneously. We further proposed that negative cooperativity (flipping LTP into LTD) could be explained by *threshold depletion* of

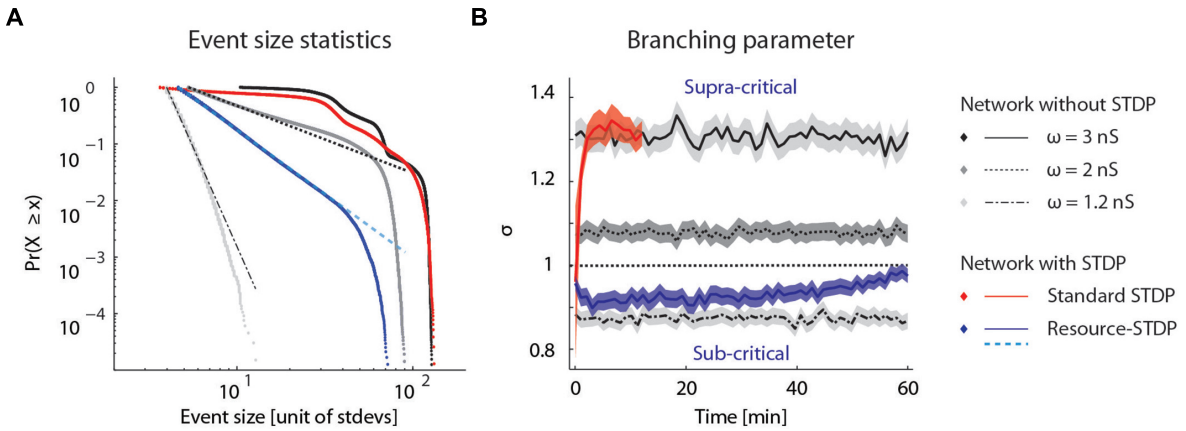

FIGURE 7 | Self-organized criticality emerges from resource-dependent STDP. (A) Cumulative probability distribution of network burst magnitudes (Clauset et al., 2009). In networks without STDP, varying the mean synaptic weight of excitatory–excitatory connections ($\omega = \bar{\omega}_{e-e}$) results in different activity regimes: sub-critical with rare aperiodic network bursts (light gray, $\omega = 1.2$ nS), weakly supra-critical with periodic network bursts at rates around 1–10 Hz (dark gray, $\omega = 2$ nS) or strongly supra-critical with periodic network bursts at high rates (black, $\omega = 3$ nS). A standard STDP rule drives the network to a strongly super-critical regime (red), whereas for resource-dependent STDP the resulting burst amplitude statistics follow a power-law (blue). Dashed lines show power-law fits to respective datasets (dot-dashed, $\omega = 1.2$ nS; dashed, $\omega = 2$ nS; cyan-dashed, resource-STDP). **(B)** The branching parameter (σ) is shown for all networks in (a). In networks without STDP, σ does not evolve (gray traces, $\omega = 1.2$, 2, and 3 nS). With a standard STDP rule, σ increases as the network becomes super-critical (red), whereas with the resource-dependent STDP rule a transition from supra-critical to (sub-) critical occurs rapidly and σ further converges toward a value around 1 (blue).

resources (opposite directions of plasticity) for LTP, such as extracellular calcium levels (Egelman and Montague, 1999). Together, these data suggest a novel mechanism for embedding local timing rules for synaptic plasticity at individual synaptic pathways into global timing rules for synaptic plasticity in the network.

One caveat of the experimental approach here is that severed neuromodulatory axons remaining in the slices could be evoked to release due to MEA stimulation, and could mediate the observed interactions between the two plasticity induction protocols. Recent advances in optogenetic stimulation methods, which can differentially target pyramidal, inhibitory, and neuromodulatory axon populations, could be employed to clarify their role. Also, applying a standard suite of inhibitors for characterizing the signaling pathways involved could further elucidate the biophysical mechanisms at play and could be pursued in follow-up studies as the basis for more detailed biophysical models.

A model in which the negative cooperativity was implemented as resource-dependent STDP was found to homeostatically regulate synaptic weights in an active network, consistent with previous observations of synaptic down-scaling in disinhibited networks (Turrigiano et al., 1998). Stable biological distributions of synaptic weights are the result, even in the presence of synchronous network activity. Moreover, network burst amplitude statistics were power-law distributed reflecting self-organized criticality, a state optimal for information coding (Bak et al., 1988; Beggs and Plenz, 2003; Shew and Plenz, 2013). Self-organized criticality has been observed in various states of vigilance *in vivo* (Petermann et al., 2009; Priesemann et al., 2009, 2013; Hahn et al., 2010), but a plasticity rule to achieve and preserve such a state has thus far been missing. The NTDP rule proposed here offers a candidate solution, and may have implications for the mechanisms underlying pathological network states that occur in epilepsy (Trasande and Ramirez, 2007) as well as the down-scaling of synaptic weights during slow-wave sleep (Massimini and Amzica, 2001; Tononi and Cirelli, 2006; Vyazovskiy et al., 2008).

The present experiments indicate that burst- and STDP$^-$-induced LTD share expression mechanisms. The proposed resource-dependent STDP model accounts for LTD at low frequencies of network bursting (Stanton and Sejnowski, 1989; Dudek and Bear, 1992) while preserving the spike-timing dependence of the underlying STDP rule, thus unifying the two phenomena under one mechanism.

Candidate mechanisms for the observed positive cooperativity rely on variables local to the dendrite (Levy and Steward, 1983; Lisman, 1989; Sjöström et al., 2001; Sjöström and Häusser, 2006). An exploration of the impact of combined negative and positive cooperativity, saturation, and blocking effects on the interaction between burst-induced plasticity and STDP would therefore require simulations of detailed neuron morphology and a biophysical STDP rule. Combined with an experimental characterization of the interactions between STDP and LTP induced by high frequency tetanic stimulation (Bliss and Lomo, 1973; Lynch et al., 1983), this approach could reveal a complement to our proposed resource-dependence of STDP, and provide a unifying model for both directionalities of burst induced plasticity, STDP, and their interactions.

Author Contributions

Experiments were conducted by VD. The resource model was designed and implemented by EM, VD, and MP. Network modeling was performed by EM and MP. System criticality analysis was performed by VD and EM. This manuscript has been written by VD, EM, DK, and HM.

Acknowledgments

We would like to thank W. Gerstner, I. Segev, W. Senn, D. Plenz, V. Priesemann, M.-O. Gewaltig, S. Ramaswamy, G. Chindemi, R. Walker, R. Perin, and S. Muralidhar for comments and helpful discussions. This work was supported by the EPFL Blue Brain Project Fund and the ETH Board Funding to the Blue Brain Project (VD, EM, DK, and HM), the Asterion Foundation (VD), and a Whitaker International Fellowship (MP).

Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fncel.2015.00220/abstract

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
published: 28 April 2015
doi: 10.3389/fncel.2015.00164

The dependence of neuronal encoding efficiency on Hebbian plasticity and homeostatic regulation of neurotransmitter release

Faramarz Faghihi[1] *and Ahmed A. Moustafa*[2,3]*

[1] *Center for Neural Informatics, Structures, and Plasticity, Krasnow Institute for Advanced Study, George Mason University, Fairfax, VA, USA,* [2] *Department of Veterans Affairs, New Jersey Health Care System, East Orange, NJ, USA,* [3] *School of Social Sciences and Psychology and Marcs Institute for Brain and Behavior, University of Western Sydney, Sydney, NSW, Australia*

Synapses act as information filters by different molecular mechanisms including retrograde messenger that affect neuronal spiking activity. One of the well-known effects of retrograde messenger in presynaptic neurons is a change of the probability of neurotransmitter release. Hebbian learning describe a strengthening of a synapse between a presynaptic input onto a postsynaptic neuron when both pre- and postsynaptic neurons are coactive. In this work, a theory of homeostatic regulation of neurotransmitter release by retrograde messenger and Hebbian plasticity in neuronal encoding is presented. Encoding efficiency was measured for different synaptic conditions. In order to gain high encoding efficiency, the spiking pattern of a neuron should be dependent on the intensity of the input and show low levels of noise. In this work, we represent spiking trains as zeros and ones (corresponding to non-spike or spike in a time bin, respectively) as words with length equal to three. Then the frequency of each word (here eight words) is measured using spiking trains. These frequencies are used to measure neuronal efficiency in different conditions and for different parameter values. Results show that neurons that have synapses acting as band-pass filters show the highest efficiency to encode their input when both Hebbian mechanism and homeostatic regulation of neurotransmitter release exist in synapses. Specifically, the integration of homeostatic regulation of feedback inhibition with Hebbian mechanism and homeostatic regulation of neurotransmitter release in the synapses leads to even higher efficiency when high stimulus intensity is presented to the neurons. However, neurons with synapses acting as high-pass filters show no remarkable increase in encoding efficiency for all simulated synaptic plasticity mechanisms. This study demonstrates the importance of cooperation of Hebbian mechanism with regulation of neurotransmitter release induced by rapid diffused retrograde messenger in neurons with synapses as low and band-pass filters to obtain high encoding efficiency in different environmental and physiological conditions.

Keywords: retrograde messenger, neurotransmitter release, homeostatic regulation, neural communication, Hebbian mechanism

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Edited by:
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Reviewed by:
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***Correspondence:**
Ahmed A. Moustafa, School of Social Sciences and Psychology and Marcs Institute for Brain and Behavior, University of Western Sydney, 2 Bullecourt Avenue, Sydney, NSW, Australia
a.moustafa@uws.edu.au

Received: *04 February 2015*
Accepted: *14 April 2015*
Published: *28 April 2015*

Citation:
Faghihi F and Moustafa AA (2015) The dependence of neuronal encoding efficiency on Hebbian plasticity and homeostatic regulation of neurotransmitter release. Front. Cell. Neurosci. 9:164. doi: 10.3389/fncel.2015.00164

Introduction

Neurons as the computational engines of the brain communicate with other neurons via synapses as conveyers of information. Neuronal firing and synaptic transmission between neurons form the building blocks for coding, processing, and storage of information in the brain (Salinas and Sejnowski, 2001). The spiking of a neuron in response to a stimulation by inputs is expected to be non-random (random means spontaneous spiking or noise) and to be dependent on input intensity. Therefore, the spiking pattern of a neuron conveys high levels of information about its inputs (high encoding efficiency) when the noise in the spiking pattern is minimized while the variation in spiking is maximized. On the other hand, low encoding efficiency is gained when a spiking pattern show low variability or high levels of noise (van Steveninck et al., 1997; Onken et al., 2014). Generally speaking, neural encoding efficiency is decreased when firing rate of a neural response is either high or very low which leads to a low variability in neural response. A low variation in a spiking pattern causes a low efficiency of encoding of either different stimuli or different intensity of a given stimulus presented to the neural system. Moreover, the diversity of neural spiking strongly depends on the properties of their synapses which remarkably vary in different types of neurons. This quantity is usually calculated using "mutual information" between a spike train and the stimulus as an information theoretic approach (Kumbhani et al., 2007; Faghihi et al., 2013; Fan, 2014; Jung et al., 2014). As neural systems should be able to detect a fluctuation in a stimulus intensity, a new encoding efficiency measure has been recently introduced which uses the geometric distance between stimulus and the response of a given neuron (Faghihi and Moustafa, 2015).

The increased complexity of synaptic protein networks was recently put forward as a potential correlate of mammalian cognitive abilities (Bayés et al., 2012; Nithianantharajah and Hannan, 2013). The diversity of synaptic plasticity mechanisms and their induced operation timescales suggest that synapses have complicated roles in information processing (Citri and Malenka, 2008; Lee et al., 2014; Yates, 2014). A long-term change in a synaptic structure provides a physiological substrate for learning and memory, whereas short-term changes support synaptic computations (Ziegler et al., 2015). The effect of an action potential transmitted from one neuron to another depends on the history of neural activity at either or both sides of the synapses such that their effect can last from milliseconds to months (Tetzlaff et al., 2012).

The release of a neurotransmitter as the main information transfer between neurons is a highly regulated process (Benfenati, 2007; Davis and Müller, 2014). Recently, neuroscience research has focused on the mechanisms of neurotransmitter release and their role in information encoding by neurons and neural network activity (Hardingham et al., 2013; Lazarevic et al., 2013; Kaeser and Regehr, 2014). Moreover, neurotransmitter release is not assured in response to synaptic stimulation, meaning that the process of neurotransmitter release in response to an action potential is essentially probabilistic.

Synapses are considered as filters that selectively and unreliably filter the flow of information between pre-and postsynaptic sites. Different synapses can show a variation in the initial probability of neurotransmitter release. Initial probability implies that the release probability may change over time. Regarding the concept of filtering, synapses are divided to three classes. For the majority of synapses in the central nervous system, the release probability at a defined synaptic contact is below 0.3, referred to as "reliably unreliable" release mechanism (Goda and Südhof, 1997). This kind of synapse is called a "high-pass filter" which is found for example in parallel fiber synapses. The synapses with high initial probability of neurotransmitter release, such as climbing fiber synapses, are called "low-pass filters" (Silver et al., 2003; Foster and Regehr, 2004; Murphy et al., 2004). Synapses with an intermediate release probability for example Schaffer collateral synapses are called "band-pass filters" (Abbott and Regehr, 2004; Rose et al., 2013).

Moreover, release probability is highly a dynamic process; it incorporates several forms of short-term plasticity mechanisms. The efficacy of synaptic transmission is dependent on the pattern of synaptic activation and the overall activity level of single neurons in a neural network. Activity-dependent changes in synaptic transmission arise from a large number of mechanisms known as synaptic plasticity (Abbott and Nelson, 2000; Lewis, 2014; Takeuchi et al., 2014; Welberg, 2014).

Functional synaptic plasticity includes homeostatic feedback mechanisms which enable neurons to respond to prolonged alterations in neuronal activity by regulating cellular excitability (Davis, 2006).

Investigating the complexity of homeostatic regulation of single neurons and neural circuits is thus fundamental for understanding brain function. Homeostatic signaling systems are thought to stabilize neural function through the regulation of ion channel density, neurotransmitter receptor abundance, and presynaptic neurotransmitter release (Davis, 2006, 2013; Marder and Goaillard, 2006; Bergquist et al., 2010; Thalhammer and Cingolani, 2014).

Homeostatic plasticity mechanisms are employed by neurons to alter membrane excitability and synaptic strength to adapt to changes in network activity. A number of cellular and molecular mechanisms have been identified as regulators of homeostatic plasticity (Maffei et al., 2012). Intrinsic membrane properties (intrinsic plasticity) as non-synaptic factors directly affect the probability that a neuron will spike in response to excitatory synaptic inputs (Kourrich et al., 2015). Based on the information of the underlying cellular mechanisms, neuronal homeostasis is categorized as the homeostatic control of intrinsic excitability of neurons by a change in ion channel expression (Turrigiano, 2011), synaptic efficacy, presynaptic neurotransmitter release, and network activity through regulation of inhibitory synapses (Turrigiano and Nelson, 2000, 2004; Davis, 2013).

Both excitatory and inhibitory synapses are subject to homeostatic regulation, and the form of plasticity present at a particular synapse likely depends on its function within a neuronal circuit. Feed-back inhibition and feed-forward inhibition as neural mechanism at network level may contribute to controlling input-output relationships in all parts of the brains (Tepper et al., 2008; Wang et al., 2013; Brown et al., 2014; Roux and Buzsáki, 2015) such that an impairment in their functionality

may be associated with some mental disorders (Phillips and Uhlhaas, 2015; Ruddock et al., 2015). An important aspect of homeostatic plasticity is the dynamic interaction between excitatory and inhibitory inputs during homeostatic adaptation, as most of the studies to date have focused on either excitatory or inhibitory synapses individually. Therefore, it is highly important to study the inhibitory role of neurons in encoding efficiency of single neurons and neural populations as well.

Hebbian plasticity and homeostatic plasticity are the two major forms of activity-dependent plasticity that modify neuronal circuits (Turrigiano, 2008). Hebbian plasticity refers to plasticity that depends on the correlations between pre- and postsynaptic activity such that excitatory synapses that effectively drive a postsynaptic cell grow stronger. This is a positive feedback process that leads to synaptic instability in the absence of additional biological constraints (Turrigiano, 2008; Vitureira and Goda, 2013; Lee et al., 2014). Homeostatic plasticity is a negative feedback mechanism that typically involves non-specific scaling of all excitatory or inhibitory synapses onto a cell to oppose changes in overall activity levels. This is thought to maintain activity levels within a dynamic range and, more generally, to stabilize neuronal circuit function despite the positive feedback of Hebbian plasticity (Turrigiano, 2008). It is believed that homeostatic plasticity operates as a compensatory, negative feedback mechanism to maintain network stability (Turrigiano, 2008; Pozo and Goda, 2010). However, it is not fully known how these two forms of plasticity interact in biological systems (Shepherd and Huganir, 2007; Turrigiano, 2008, 2011; Vitureira and Goda, 2013). In models that combine Hebbian plasticity with homeostatic plasticity, homeostatic plasticity generally stabilizes a set of unsaturated weights that would be unstable under Hebbian plasticity alone (Toyoizumi et al., 2013, 2014). However, such stabilization fails if homeostatic plasticity is too slow compared to unstable Hebbian plasticity (Zenke et al., 2013). This is an example of the more general result that slow negative feedback cannot stabilize a fast, unstable positive feedback process. Some modeling studies have shown that long-term changes in synaptic weights are difficult to achieve without a "normalizing" mechanism to regulate total synaptic strength or excitability (Pérez-Otaño and Ehlers, 2005; Shepherd and Huganir, 2007; Newpher and Ehlers, 2008). The role of some chemicals as retrograde messengers in regulating presynaptic neurotransmitter release has been previously shown (Yang and Calakos, 2013; Zachariou et al., 2013; Nadim and Bucher, 2014; Padamsey and Emptage, 2014).

Diffusible messengers that have been previously implicated in activity-dependent presynaptic changes are plausible candidates also for homeostatically adjusting presynaptic release properties according to dendritic activity (Jakawich et al., 2010; Lindskog et al., 2010; Ohno-Shosaku et al., 2012).

In particular, endocannabinoids have been shown to function as retrograde messengers at CNS synapses (Castillo et al., 2012). The importance of retrograde messengers (e.g., nitric oxide, arachidonic acid, adenosine and platelet activating factor) in Hebbian plasticity and so in homeostatic processes has been proposed (Lily and Goda, 2009; Ohno-Shosaku and Kano, 2014; Wang et al., 2014). Notably, the neurotrophin BDNF, whose role in Hebbian plasticity is well established, has also been shown to play a role in homeostatic synaptic plasticity (Liu et al., 2014; Lu et al., 2014). There are research interests about the effect of a change in neurotransmitter release machinery on homeostatic presynaptic plasticity. The investigation of how homeostatic mechanisms observed at both single neuron and circuit level are integrated to regulate brain activity is a very challenging neuroscience research topic. Answering this question is potentially important if we aim to gain a comprehensive understanding on how neural plasticity in different physiological conditions is regulated to obtain high efficiency of information processing by both single and neural populations. Slow homeostatic plasticity cannot stabilize the instability effect of Hebbian plasticity. Therefore, exploring multiple regulatory pathways of interaction of these two plasticity mechanism which operate at different timescales is required to understand how they help brain to encode information (Turrigiano, 2012; Toyoizumi et al., 2014). To understand the cooperation of synaptic and non-synaptic mechanisms which operate over different timescales a model has been recently presented in which neuronal information is represented as probability distributions (Tully et al., 2014).

In this work, the main objective is to study the interaction of Hebbian plasticity and retrograde signaling which has a fast rate of diffusion from post to presynaptic sites of neurons, and influence regulation of neurotransmitter release. For this purpose, neurons with synapses that act as different information filters are simulated. The neuron's efficiency to encode its input when a different level of stimulation is presented to the neural system is measured. Hebbian mechanism and homeostatic regulation of neurotransmitter release by retrograde messenger are modeled in the synapses of 1000 neurons fully connected into a neural population where the synaptic dynamic of a single neuron of the population is studied. The model uses known basic information about biochemical interactions underlying the production of fast diffused retrograde messenger and hypothetical neurotransmitter release inhibitory machinery which is affected by pre-and postsynaptic activities. This hypothetical complex in real neurons may be composed of some protein-protein interactions or the activity of a multi-subunits protein which is activated by independent or dependent pathways. The motivation of such complex mechanisms for inhibiting neurotransmitter release is the increased evidence that supports the role of different proteins and biochemical pathways in neuronal activities. The effect of each individual mechanism and in combination with each other is studied. Moreover, the effects of integrating the homeostatic regulation of feedback inhibition by inhibitory neuron with modeled synapses on encoding efficiency of single neurons are studied. In the next sections, the details of the dynamic model and the simulations are presented. The overall importance of the results is addressed in discussion.

Materials and Methods

Model Architecture

A neuron in biological neural systems receives a large number of spikes from other neurons via synapses. These spikes are then integrated and transmitted by generating spike trains to other

neurons. Such activity should be stable and efficient to transfer information. In order to simulate such complicated information processing mechanism by a single neuron, we consider a feed-forward neural layer composed of 1000 neurons fully connected to the second neural layer (100 neurons) in which we measure single neurons' encoding efficiency. Each neuron in the second layer is connected via a single synapse to neurons in the second layer. The neural activity of each of 1000 neurons is modeled as a probability of generating a spike in each time bin equal to 10 ms. The firing probability of neurons demonstrates the stimulus intensity detected by the neural system. The intensity of input to neurons may vary in different environment where the intensity of a stimulus changes over time. Therefore, it is highly vital to be able to encode fluctuating environmental stimuli by brains.

TABLE 1 | Parameters of the integrate and fire neuron model used in this study.

Parameter	Value
V_{rest} resting potential	−84 mV
V_{thresh} threshold of spiking	−25.8 mV
V_{recov} recovery threshold	−40.2 mV
V_{spike} spike potential	9.5 mV
g_{leak} membrane conductance	0.26 nS
k membrane capacitance	4.0 pF

The spiking activity of single neurons was modeled using an integrate and fire model (Equation 1).

$$k\frac{dV}{dt} = -g_{leak}\,(V - V_{rest}) + \sum I(t) \tag{1}$$

Where $\sum I\,(t)$ is the sum of input currents from the presynaptic neurons into postsynaptic site of a single neuron.

Table 1 shows the electrophysiological parameters used in the study (Wüstenberg et al., 2004).

Synaptic Modeling

A synapse between neurons is modeled using simplified known mechanistic events (**Figure 1**). When an action potential reaches the presynaptic site it leads to an increase in intercellular calcium in the presynaptic site which consequently activates some biochemical pathways by activating proteins and protein-complexes. An action potential may also lead to neurotransmitter release which causes current into the postsynaptic site. In this work, it is assumed that the current into the postsynaptic site induces the production of retrograde messenger which is diffused rapidly into the presynaptic site. Moreover, the sum of currents into the postsynaptic site triggers any single neuron to generate spikes train according to integrate and fire neuron (Equation 1). Retrograde messenger can trigger some pathways that interact with activated proteins in presynaptic neuron to activate a

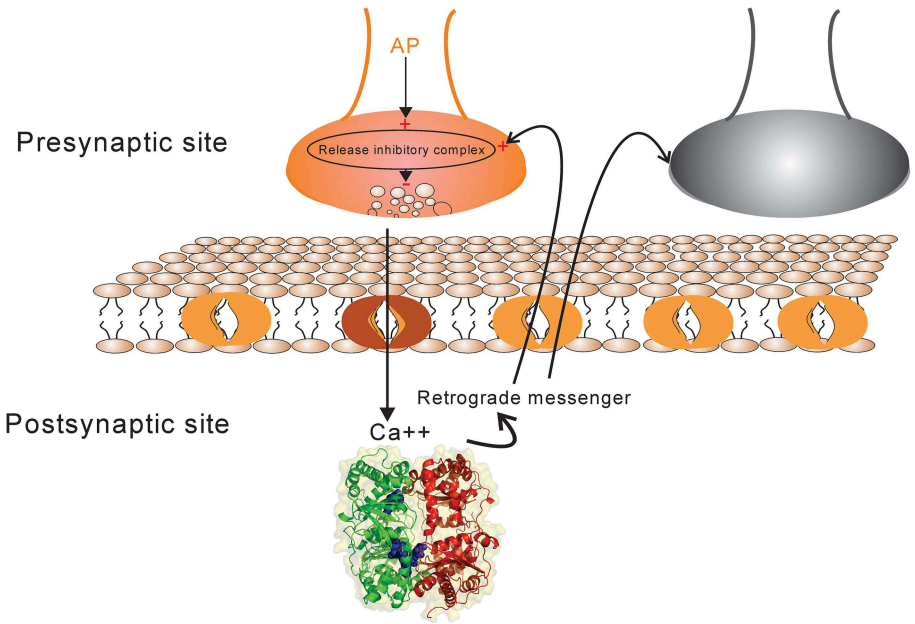

FIGURE 1 | The schematic of synapse model. Action potential in each activated synapse (presynaptic site shown by orange) may lead to neurotransmitter release according to the initial probability of release. Modified release probability induced by the activity of release inhibitory complex can change the pattern of neurotransmitter release which induces current into the postsynaptic site. The current influx is also affected by synaptic efficacy which is determined directly by presynaptic spike trace and indirectly by Hebbian mechanism. The retrograde messenger is produced in the postsynaptic site and is released into the presynaptic site where it affects release probability complex. The sum of current influx into the postsynaptic sites triggers a single neuron to generate a spike train in response to a stimulus presentation to the neural population (presynaptic neurons). The spiking pattern of pre-and postsynaptic neurons can modify presynaptic neuron's activity and synaptic efficacy between pre-and postsynaptic neurons as well by a Hebbian mechanism.

complex that may inhibit neurotransmitter release with different probabilities.

Hebbian synaptic plasticity is modeled in synapses such that its effect on a single neuron efficiency in combination with neurotransmitter regulation or independently is studied.

In each simulation study, a random set of neurons of 1000 neuron population is activated with a firing probability. The number of activated neurons is extracted according to Gaussian distribution with mean equal to 500 and variance equal to 50.

The current influx into the postsynaptic site is modeled as Equation (2).

$$I(t) = \omega \left(\frac{t}{\tau}\right) e^{\frac{-t}{\tau}} \sum_{tp} \delta\left(t - tp\right) \quad \tau = 10\,ms \tag{2}$$

Where τ is the decay rate of current and ω is the synaptic weight between each activated single neuron in the second layer and a neuron in the first layer. $\sum_{tp} \delta\left(t - tp\right)$ is the Dirac function.

The activity trace of spike in the presynaptic site was modeled using Equation (3).

$$\dot{C} = \frac{-1}{\tau_c}\left(C + \triangle \sum_{tp} \delta\left(t - tp\right)\right) \tau_c = 100\,ms \tag{3}$$

The change of synaptic efficacy is modeled using Equation (4).

$$\dot{\omega} = Cd \tag{4}$$

Where d is the dopamine level in each time bin which is generated by high firing rate of dopaminergic neuron (equal to 0.9) (Equation 5).

$$\begin{aligned} \dot{d} &= \frac{-1}{\tau_d}\left(d + \sum_{t_d} \delta\left(t - t_d\right)\right) \\ \tau_d &= 20\,ms \end{aligned} \tag{5}$$

To model Hebbian synaptic plasticity we used a simple rule shown in **Table 2**.

Table 2 shows that when spiking of a neuron in the first layer is followed by spike in a neuron in the second layer, it may lead to higher synaptic efficacy between pair of neurons according to Equations (3) and (4).

Total produced retrograde messenger (RM) at the end of each time bin is generated according to Equation (6) such that just high levels of current can generate effective levels of retrograde messenger.

$$RM = \frac{\left(\sum I\right)^2}{1 + \left(\sum I\right)^2} \tag{6}$$

TABLE 2 | Hebbian learning rule used in the model.

Presynaptic state	1	1	0	0
Postsynaptic state	1	0	1	0
△	1	−1	−1	0

It is assumed that at the end of each time bin retrograde messenger is accumulated in the presynaptic site and its dynamics is modeled using Equation (7).

$$\begin{aligned} \dot{RM_{trace}} &= \frac{-1}{\tau_r}\left(RM_{trace}\right) + RM \\ \tau_r &= 400\,ms \end{aligned} \tag{7}$$

The activity of the complex which may inhibit neurotransmitter release (R_{inh}) is modeled as Equation (8).

$$\begin{aligned} \dot{R_{inh}} &= \frac{-1}{\tau_{inh}}\left(R_{inh}\right) + RM_{trace}C \\ \tau_{inh} &= 200\,ms \end{aligned} \tag{8}$$

Equation (8) shows that the activity of hypothetical complex to inhibit neurotransmitter release depends directly on the concentration of retrograde messenger in the presynaptic site and spike trace in the presynaptic site. To model probability of neurotransmitter release inhibition by inhibition-complex activity, we assume that this probability is changed such that higher activity can lead to higher probability (Equation 9). We assume that α should be decreased when the activity is raised (Equation 10). Hence, the probability of neurotransmitter release as a function of complex activity is presented as Equation (11) and is shown in **Figure 2**.

$$P_{inh} = e^{\frac{-\alpha}{R_{inh}}} \tag{9}$$

$$\alpha = 1 - e^{\frac{-0.1}{R_{inh}}} \tag{10}$$

Hence,

$$P_{inh} = e^{\frac{\frac{-0.1}{\left(e^{R_{inh}-1}\right)}}{R_{inh}}} \tag{11}$$

$$P_{rel} = \left(1 - P_{inh}\right).P_{init} \tag{12}$$

Equation (12) shows the relationship between release probability (P_{rel}) and inhibition probability (P_{rel}) regarding initial neurotransmitter release probability (P_{init}). In order to investigate the role of different kind of synapses in information processing by neurons, it is highly important to examine different initial release probability. Therefore, three initial release probabilities were considered as 0.25, 0.55, and 0.85 for synapses as high, band and low pass filters, respectively.

A challenge in modern neuroscience is how to measure the efficiency of a neural population to encode information that is received by neurons and is encoded as spiking patterns. For this purpose, information theory has proposed some measures including mutual information that can be measured by different approaches to study the role of structural and physiological parameters involved in neural systems of different senses (van Steveninck et al., 1997). Recently, a geometrical approach has been introduced that aims to measure neural system efficiency by the calculation of defined words in a given neural response (Faghihi and Moustafa, 2015). In the current study, this approach

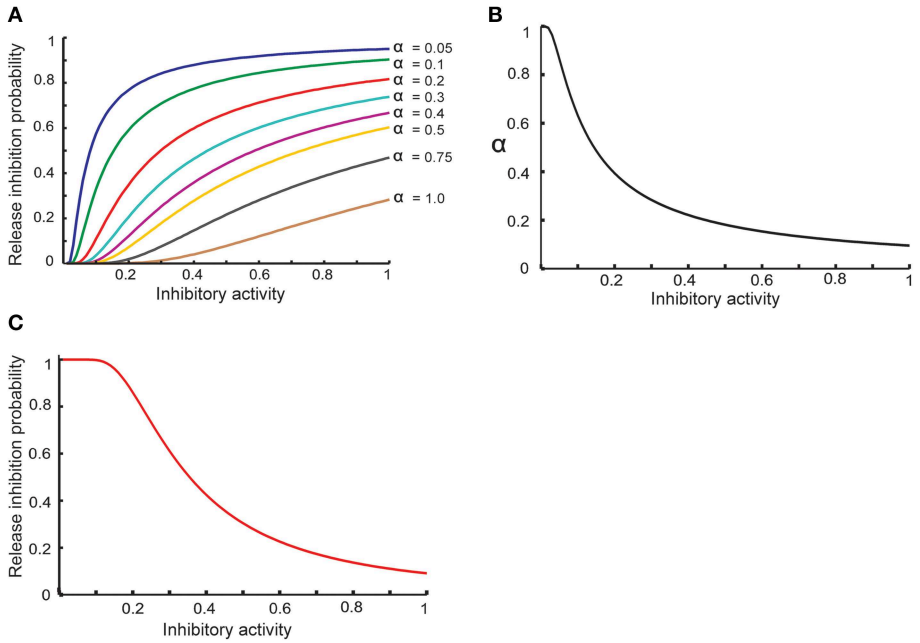

FIGURE 2 | Modeling homeostatic regulation of neurotransmitter release. (A) The relationship between the inhibitory complex activity and release inhibition probability for different parameter values between 0.05 and 1. The plot demonstrates a non-linear relationship between inhibitory complex activity and release inhibition probability for different model parameter (α) values. Higher inhibitory complex activity needs a lower α value while low inhibitory complex activity needs a higher α value. These observations motivated to define an exponential relationship between inhibitory complex activity and α value **(B)** which lead to non-linear relationship of inhibitory complex activity and release inhibitory probability **(C)**.

is used to measure the role of different parameters including different synapses in a single neuron's efficiency to encode its inputs. For this purpose, words composed of zeros and ones corresponding to non-spike and spike in each time bin, respectively, are defined with length equal to three. The frequency of each word in the spiking train is calculated such that any spiking train is represented as an ordered vector with length equal to eight. The stimulus intensity as firing probability of neurons in the first layer is changed 5% for probabilities from 0.5 to 0.95. For probability of firing equal to 1, the firing probability is decreased 5%. The spiking train of a single neuron in the second layer represented as vectors are used to measure the distance of neural responses as a measure how the spiking has encoded the fluctuation in its input.

Results

Stimuli with different intensities were presented to the input layer as different firing probability of neurons in neural population (1000 neurons connected fully to second neural layer). The input layer triggers neurons in the second layer to spike with different frequency that depends on model's synaptic or network parameters. For high initial release probability of synapses between the first and second neural layer, the efficiency of a neuron of the second layer to encode stimulus information was measured for different parameter values and different assumptions about synaptic mechanisms. To model encoding efficiency of a single neuron in the second layer as a function of synaptic mechanisms and neural architectures, the inhibition of neurotransmitter release plays a critical role in this study.

Figure 2A shows the basic assumption about the relationship between release inhibitory activity in the presynaptic neuron and release inhibition probability. α value determines the dependency of release probability on level of inhibitory activity. In order to define a homeostatic regulation of release inhibition probability by inhibitory activity, the model assumes that a decrease in α value when inhibitory activity is raised. **Figure 2B** shows the relationship between α value and inhibitory activity in this study. Hence, the modeled relationship between inhibitory activity and release probability (Equation 11) is illustrated in **Figure 2C**. By homeostatic change of release probability, in different synaptic conditions and different assumptions used in the study, encoding efficiency of a single neuron was measured.

Figure 3 shows the change of the models' parameter values of a neuron with synapses as a low pass filter (initial release probability equal to 0.85) in the presence of Hebbian mechanism without modeling homeostatic regulation of neurotransmitter release. In the absence of retrograde messenger production in the postsynaptic site, no change in neurotransmitter release is induced in the presynaptic site in the time bin between 50 and 150. In the other time bins, a single neuron spikes in the absence of both mechanisms. **Figure 3A** shows a presynaptic neuron spiking when a high stimulus intensity was presented to the first neural layer as firing probability equal to 0.85. This spiking pattern is used in all simulations in order to compare the parameter values in different conditions. The existence

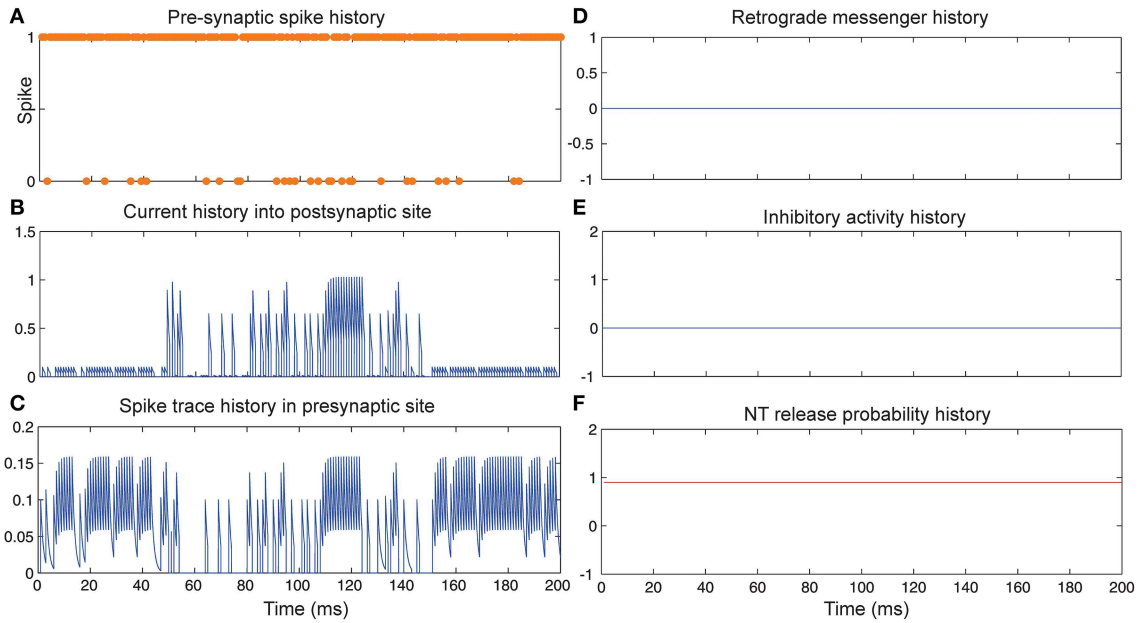

FIGURE 3 | The dynamics of model's parameters in the presence of Hebbian mechanism and absence of homeostatic regulation of neurotransmitter release. (A) Spike train history of a presynaptic neuron with firing probability equal to 0.85 in 200 time bins. **(B)** Current history into the postsynaptic site triggered by presynaptic neuron's spiking. In time bins between 50 and 150 Hebbian rule was applied to the model while in the postsynaptic site production of retrograde messenger was blocked. **(C)** Spike trace history in the presynaptic site in time bins between 50 and 150. Spike trace activity is affected by both spiking pattern of presynaptic neuron and Hebbian mechanism. **(D–F)** No change in retrograde messenger concentration in the presynaptic site is observed. This leads to very low activity in inhibitory complex (equal to zero) and consequently in neurotransmitter release probability (equal to initial release probability).

of Hebbian mechanism leads to high levels of current into the postsynaptic neuron (**Figure 3B**). Hebbian mechanism also induces changes in spike trace activity in the presynaptic neuron in time bins between 50 and 150 (**Figure 3C**). In the absence of retrograde messenger production in the postsynaptic site, no changes in release inhibitory activity in presynaptic site are observed (**Figures 3D–F**). These activities of neurons in the first layer result in a spiking train of neurons in the second layer. The spiking activity of a single neuron in the second layer is presented in **Figure 6B**.

Figure 4 shows the change of model's parameter values of a neuron with synapses as low pass filter (initial release probability equal to 0.85) in the presence of homeostatic regulation of neurotransmitter release and the absence of Hebbian mechanism in time bins between 50 and 150. Retrograde messenger is produced by the postsynaptic neuron in response to the presynaptic current and is diffused into the presynaptic neuron (**Figure 4D**) which leads to an increase of inhibitory activity in the presynaptic neuron (**Figure 4E**). Consequently, it leads to changes in neurotransmitter release probability (**Figure 4F**). Overall activities of the presynaptic neurons induce changes in: spiking activity of the presynaptic neuron (**Figure 4A**), current influx into the post-synaptic neuron (**Figure 4B**) and spike trace induced activity (**Figure 4C**). The spiking activity of a single neuron in the second layer is presented in **Figure 6C**.

Figure 5 shows the change of model's parameter values of a neuron with synapses as low pass filter (initial release probability equal to 0.85) in the presence of both homeostatic regulation of neurotransmitter release and Hebbian mechanism in the pre- and postsynaptic neurons. The current influx into postsynaptic neuron (**Figure 5B**) is affected by both mechanisms. Spike trace activity is affected by Hebbian mechanism (**Figure 5C**). The retrograde messenger level in the presynaptic neuron, inhibitory activity and neurotransmitter release probability is presented in **Figures 5D–F**, respectively. The spiking activity of a single neuron in the second layer is presented in **Figure 6C**.

Figure 6 shows that the spiking frequency of a single neuron in the second layer which is highly dependent on the synaptic mechanisms in the simulations. Hebbian mechanism in the absence of controlling of neurotransmitter release leads to a very high firing rate in single neurons in the second layer (**Figure 6B**). In the presence of inhibition of neurotransmitter release while the Hebbian mechanism was blocked, a lower firing rate of a single neuron in the second layer is observed (**Figure 6C**). When the model included both Hebbian and neurotransmitter release mechanisms, simulations show moderate firing rates in the spiking trains of a single neuron in the second layer (**Figure 6D**).

The main aim of this study is to measure efficiency of a single neuron in the second layer to encode its input. The efficiency measure used in this study allows the study of efficiency of a single neuron to encode fluctuation in their input as a vital capability of the animal brain to live in dynamic environments. For this purpose, the encoding efficiency was measured for

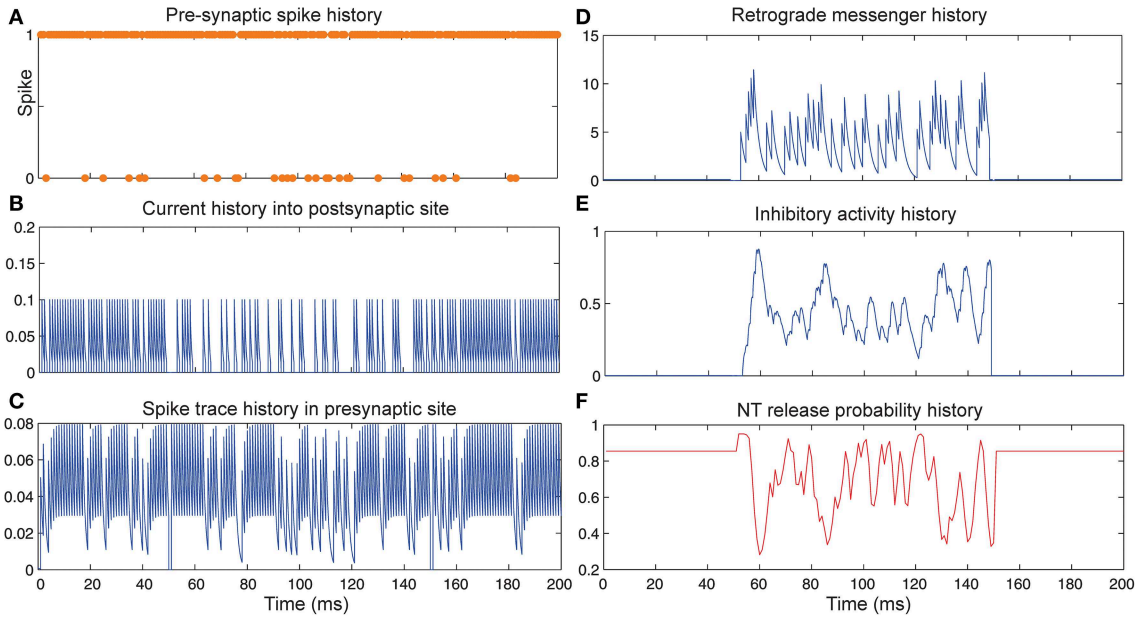

FIGURE 4 | The Dynamics of model's parameters in the presence of homeostatic regulation of neurotransmitter release and absence of Hebbian mechanism. (A) Spike train history of a presynaptic neuron with firing probability equal to 0.85 in 200 time bins. **(B)** Current history into the postsynaptic site triggered by presynaptic neuron's spiking. In time bins between 50 and 150 homeostatic regulation of neurotransmitter release was applied by generation of retrograde messenger in the postsynaptic site and its effect in presynaptic neuron. **(C)** Spike trace history in the presynaptic site in time bins between 50 and 150. Spike trace activity is affected by both spiking pattern of the presynaptic neuron. **(D)** Retrograde messenger is generated in the postsynaptic site and received by the presynaptic site as a consequence of the presynaptic neuron activity. **(E)** Inhibitory complex activity is affected by both retrograde messenger and spike trace activity in time bins between 50 and 150. **(F)** Neurotransmitter release probability is determined by the inhibitory complex activity.

different synaptic conditions at different firing probabilities of the input layer.

Figure 7A shows that for synapses that act as low-pass filters (initial neurotransmitter release probability equal to 0.85), the maximum efficiency of a single neuron is obtained when Hebbian mechanism and homeostatic regulation of neurotransmitter release are integrated and interact with each other in the model. However, in the absence of Hebbian mechanism or homeostatic regulation of neurotransmitter release the efficiency of a single neuron is found to be low. Minimum efficiency is obtained when synapses without both mechanisms are modeled.

Figure 7B shows the encoding efficiency of neurons with synapses which act as band-pass filters (initial neurotransmitter release probability equal to 0.55) for different synaptic mechanisms. The comparison of these results with neurons with synapses that act as low-pass filters revealed a higher encoding efficiency when both Hebbian plasticity and homeostatic regulation of neurotransmitter release exist in the synapses. When initial neurotransmitter release probability was set to 0.25 (synapses acting as high-pass filters) the encoding efficiency for all synaptic conditions is remarkably lower than low and band pass filters (**Figure 7C**).

The effect of feedback inhibition was studied in this work in combination with Hebbian mechanism and homeostatic regulation of neurotransmitter release. **Figure 8A** shows that for neurons with synapses acting as low pass filters (high initial release probability equal to 0.85) when high firing probability of the input layer is presented to the neural system, feedback inhibition helps a single neuron keep its efficiency at high levels. For high pass filters (low initial release probabilities set to 0.25) the association of Hebbian learning with homeostatic regulation of neurotransmitter release independently or in combination with feedback inhibition does not help neurons show high levels of encoding efficiency (**Figure 8C**). The highest encoding efficiency was obtained when encoding efficiency of neurons with synapses as band-pass filters were simulated (**Figure 8B**).

Discussion

New explorations have shown different kinds of neuronal plasticity and neuromodulations that influence neural communication. Neuromodulators can exert effects at different timescales from short term to persistent long term regulations. The temporal dynamics of neuromodulator release plays an important role in the modulation of neural circuits, yet its effect on circuit output is not easy to understand (Marder, 2012). However, it is required to explore integrative functionalities of neuromodulators and plasticity mechanisms in network dynamics. For a better understanding of the cellular event underlying short and long term neuronal plasticity and network dynamics, a new generation of models and theories is required (Doya et al., 2002; Dayan, 2012).

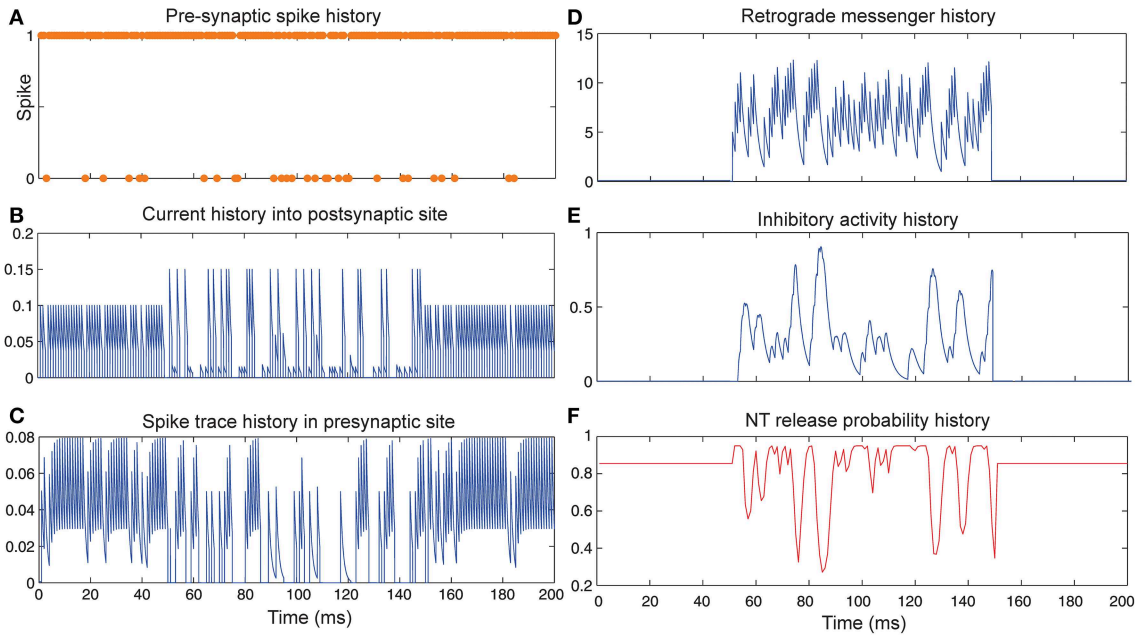

FIGURE 5 | The Dynamics of model's parameters in the presence of homeostatic regulation of neurotransmitter release and Hebbian mechanism. (A) Spike train history of a presynaptic neuron with firing probability equal to 0.85 in 200 time bins. **(B)** Current history into the postsynaptic site triggered by the presynaptic neuron's spiking. In time bins between 50 and 150 homeostatic regulation of neurotransmitter release was applied by generation of retrograde messenger in the postsynaptic site and its effect in the presynaptic site. **(C)** Spike trace history in the presynaptic site in time bins between 50 and 150. Spike trace activity is affected by both spiking pattern of the presynaptic neuron and Hebbian mechanism. **(D)** Retrograde messenger is generated in the postsynaptic site and received by the presynaptic site as a consequence of the presynaptic neuron activity. **(E)** Inhibitory complex activity is affected by both retrograde messenger and spike trace activity in time bins between 50 and 150. **(F)** Neurotransmitter release probability is determined by inhibitory complex activity.

In this work, an approach was applied to measure encoding efficiency which is based on counting the frequency of defined words in a spiking pattern. The method measures the efficiency of a neuron to detect fluctuation in its inputs.

The activity of a neuron may be affected by some mechanisms at network levels like feed-back and feed-forward inhibition such that any abnormality in these neurons may cause some mental disorders (Brown et al., 2015). Therefore, in this study the question that was addressed was how neuron encoding efficiency is determined in an integrated paradigm in which Hebbian learning rule and retrograde messenger effect on neurotransmitter release exist in synapses. Specifically, it is not known what potential roles played by inhibitory neurons that widely exist in neural systems in such complicated cellular events.

To address key questions related to the possible effect of the Hebbian learning rule and retrograde messengers on the presynaptic neurons and their role in homeostatic regulation of spiking activity, we developed a hypothesis that presume a molecular machinery which is responsible for inhibiting neurotransmitter release of the neuron. Such machinery may be a cellular pathway or a set of protein-protein interactions such that its activation depends on the effective presence of a spike trace (molecular changes induced by action potential) and induced effects of diffused retrograde messenger from postsynaptic neurons. The importance of dependency of release inhibition activity on spike trace is to prevent any non-specific activation of molecular machinery by diffused retrograde messenger to non-activated synapses in neural networks. Our modeling and simulation results suggest novel experiments to explore such molecular machinery or biochemical pathways. In this model, spiking of the presynaptic neurons (if associated with neurotransmitter release) may trigger postsynaptic neurons to produce locally retrograde messenger which is rapidly received by the presynaptic neuron. Such assumption suggests gaseous chemical like nitric oxide as a retrograde messenger candidate for this hypothesis (Hardingham et al., 2013; Neitz et al., 2014; Sagi et al., 2014). Non-gaseous chemicals may have a longer time scale to affect presynaptic neurons so their contribution in interaction with Hebbian mechanism may lead to different results. If associated with effective levels of spike trace, received retrograde messengers received by presynaptic neurons may lead to the inhibition of neurotransmitter release. A decrease in synaptic weight is obtained if the postsynaptic neuron spikes in response to the sum of its input (according to the Hebbian rule, in **Table 2**). In this study, we modeled synapses in which Hebbian plasticity and neuromodulatory mechanism as fast diffused outward of retrograde signaling exist and interact in short term timescales. The best candidate for such retrograde signaling is nitric oxide which is produced and diffused by the stimulation of the sum of input current from presynaptic sites. Such assumption gives rise to the existence of a loop between pre-and postsynaptic sites as follows: retrograde messenger from postsynaptic

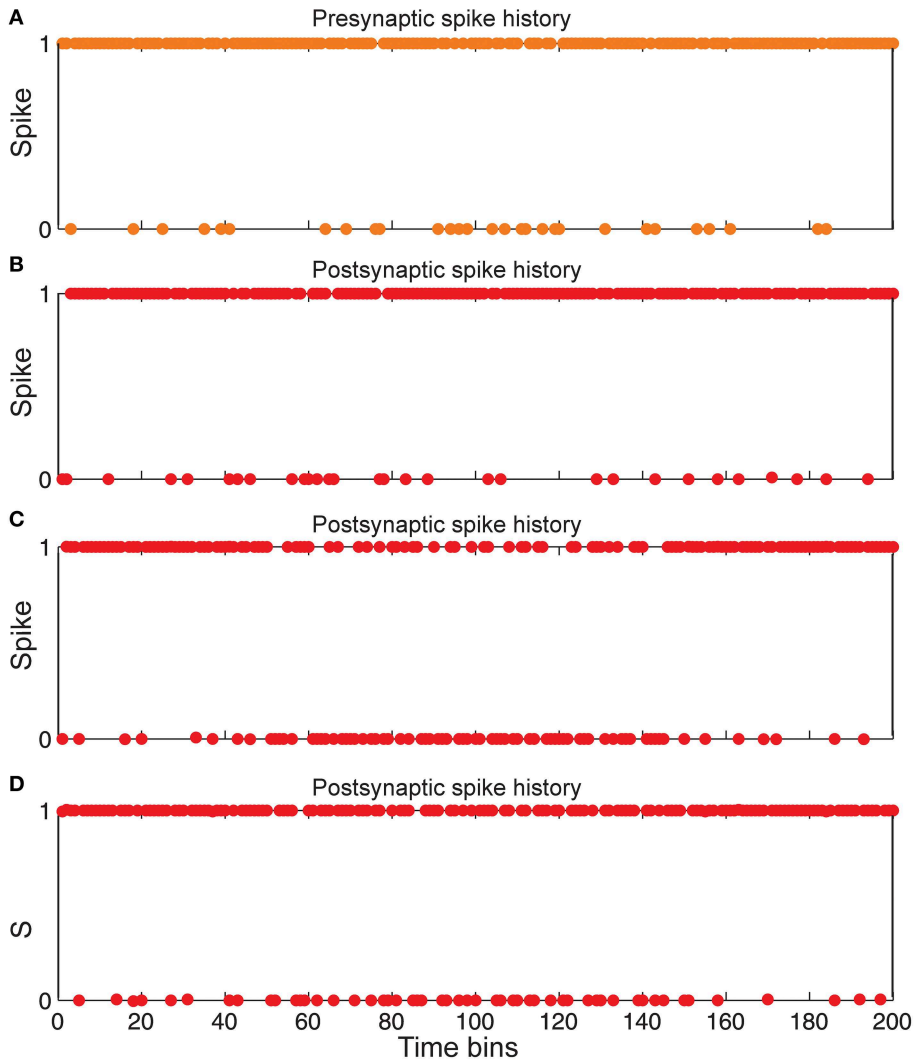

FIGURE 6 | Comparing spiking in pre-and postsynaptic neurons for different synaptic conditions. (A) Spiking history of a presynaptic neuron in response to stimulus presentation as firing probability equal to 0.85. **(B)** Postsynaptic spiking history in the presence of Hebbian mechanism and in the absence of homeostatic regulation of neurotransmitter release induced by retrograde messenger. **(C)** Postsynaptic spiking history in the absence of Hebbian mechanism and in presence of homeostatic regulation of neurotransmitter release induced by retrograde messenger. **(D)** Postsynaptic spiking history in presence of both Hebbian mechanism and homeostatic regulation of neurotransmitter release induced by retrograde messenger.

site modifies release probability of neurotransmitter. The released neurotransmitter in combination with synaptic efficacy determines the current influx into postsynaptic site. The spike timing of postsynaptic activity triggered by total current affects the Hebbian plasticity mechanism. Consequently, synaptic efficacy between pre-and postsynaptic sites is changed and so it leads to a change in current into postsynaptic site which modify retrograde messenger production in the next time bins. Hence, the combination of both mechanisms at the network level has resulted in a firing rate of postsynaptic neuron (single neuron) at moderate level (not too high or too low) when high stimulus intensity was presented to the neural system. In such stimulus presentation conditions, in the absence of retrograde messenger effect on presynaptic neuron, due to Hebbian learning rule, postsynaptic neurons generate spiking with high frequency which leads to a low encoding efficiency. Retrograde signaling in the absence of Hebbian learning rule can help neurons to control their spiking activities, but encoding efficiency does not reach high levels because release inhibitory activity is not strong enough to control spiking rate. Moreover, adding the simulation of effective inhibitory feedback by an inhibitory neuron on the network shows its vital role in encoding efficiency of single neurons when combined with Hebbian mechanism and retrograde messenger comparing to the efficiency in the absence of these synaptic mechanisms (Faghihi and Moustafa, 2015). These simulations assign a critical role for nitric oxide as a known retrograde messenger with desired properties for the proposed hypothesis. Therefore, our simulation studies provide

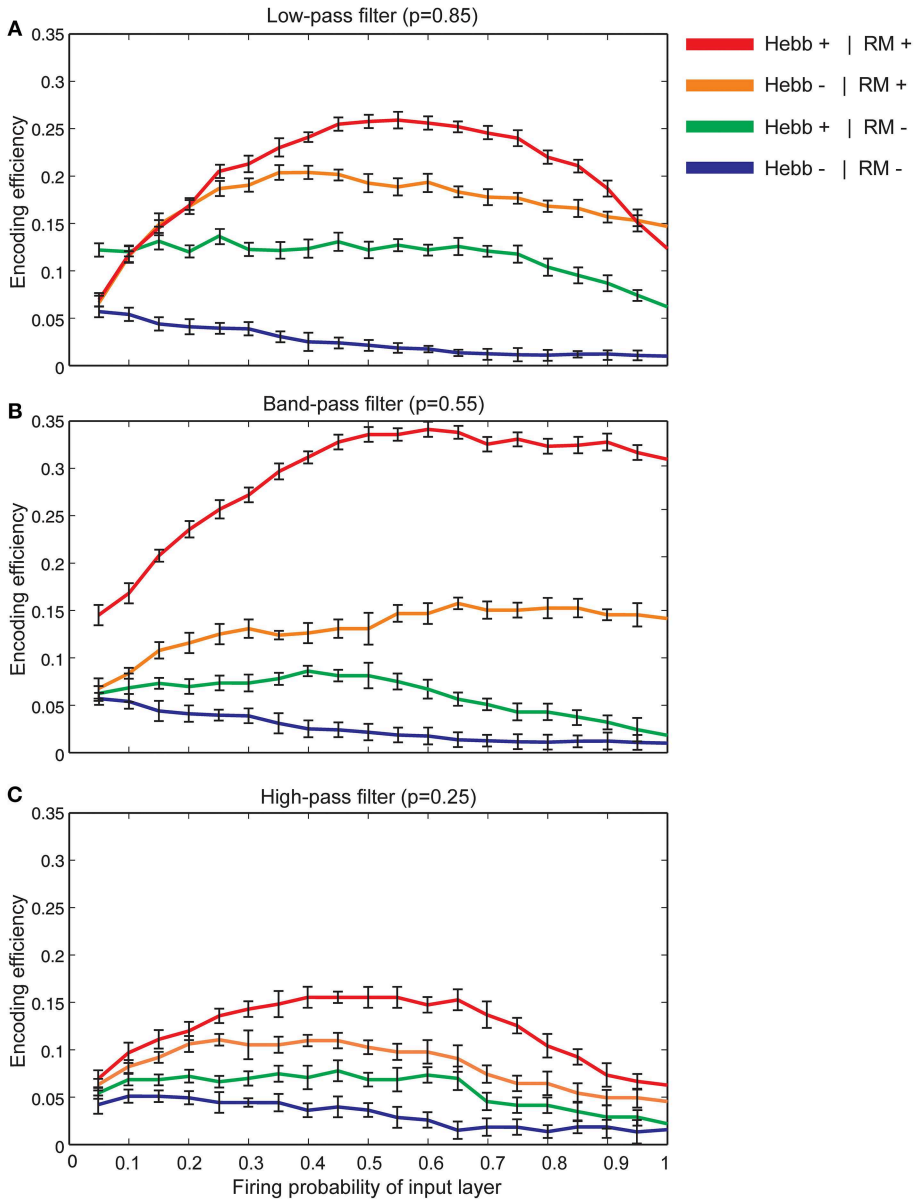

FIGURE 7 | The encoding efficiency of a single neuron in different synaptic conditions. (A) Synapses as low-pass filters. Initial release probability was set to 0.85. The maximum efficiency was observed when both Hebbian mechanism and homeostatic regulation of neurotransmitter release were modeled (red line). Homeostatic regulation of neurotransmitter release in the absence of Hebbian mechanism leads to lower efficiency in comparison to the presence of both mechanisms in the modeled synapse (brown line). The combined presence of Hebbian mechanism and the absence of homeostatic regulation of neurotransmitter release lead to a higher efficiency in comparison to efficiency in the absence of both mechanisms (shown as green and blue lines, respectively). Hebb+ stands for existing Hebbian mechanism and RM stands for existing of retrograde messenger based induced activity. **(B)** Synapses as band-pass filters. Initial release probability was set to 0.55. In comparison to low pass-filters, when both Hebbian mechanism and homeostatic regulation mechanism of retrograde messenger exist, studied a single neuron demonstrates higher encoding efficiency. **(C)** Synapses as high-pass filters. Initial release probability was set to 0.25. In comparison to low and band-pass filters, studied single neuron shows low encoding efficiency for all synaptic conditions.

new predictions and additional experiments on the role of this chemical in the nervous system.

Retrograde messenger with different timescales of operation may play other roles in homeostatic regulation of neuronal spiking stability. One may be its role as an error signal. Such error signal as the difference between the basal level of retrograde messenger or synaptic efficacy and updated level can act as a correction mechanism to stabilize synaptic activity (Davis, 2006). Therefore, the correction mechanism of different retrograde messengers in combination with Hebbian plasticity

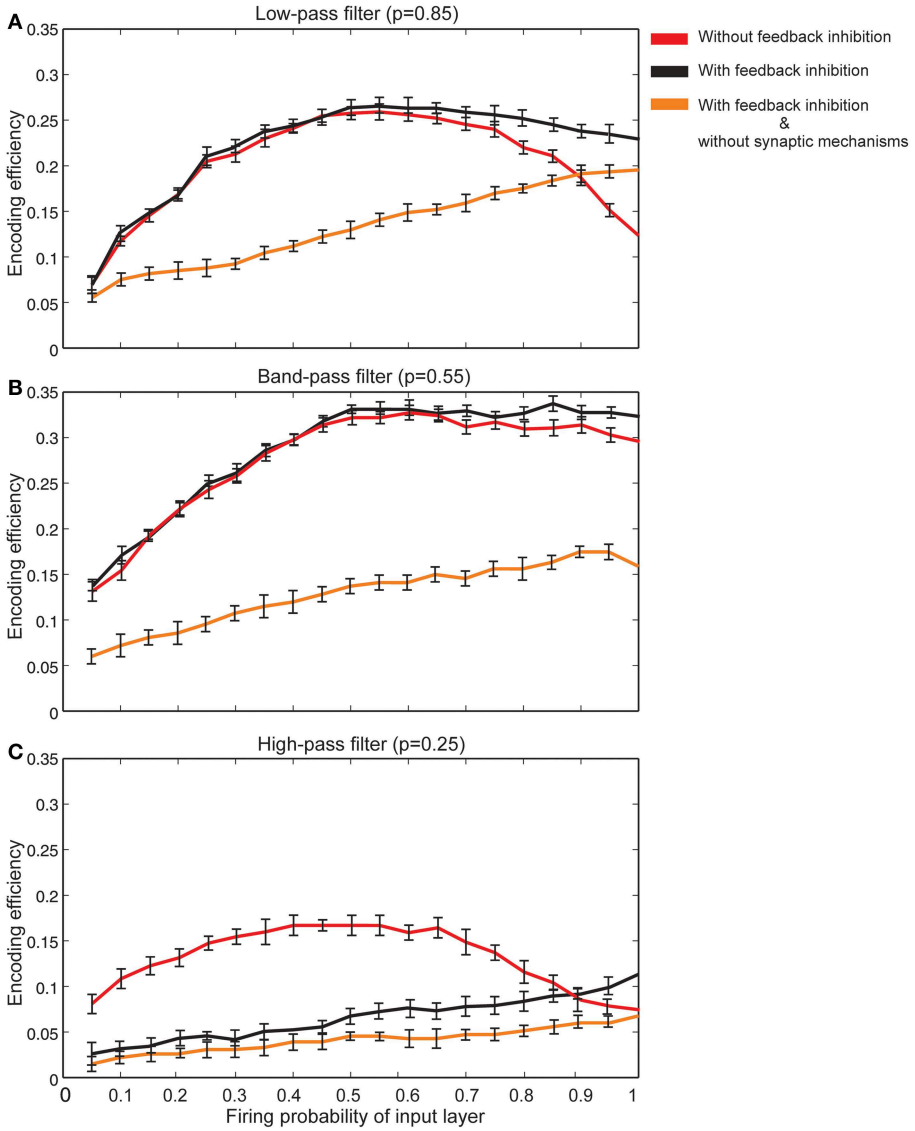

FIGURE 8 | The effect of feedback inhibition in efficiency of a single neuron for different types of synapse. (A) Synapses as low-pass filters. Initial release probability was set to 0.85. In the presence of feedback inhibitory neuron the efficiency of a single neuron to encode stimulus presented to the neural system is higher for high firing probability of input layer (for firing probability larger than 0.6). **(B)** Synapses as band-pass filters. Initial release probability was set to 0.55. In comparison to low-pass filters, in the presence of feedback inhibitory neuron the efficiency of a single neuron to encode stimulus is higher. **(C)** Synapses as high-pass filters. Initial release probability was set to 0.25.Neuron shows low levels of encoding efficiency with or without feedback inhibitory effect.

and homeostatic plasticity should be considered in future computational modeling work.

In our simulations, a high firing rate was used for dopaminergic neurons in order to keep dopamine at constant levels such that the dynamics of synaptic efficacy is affected only by changes in the spike trace and Hebbian learning rule. In future work, one may examine different levels of dopaminergic neurons' firing rate and different learning strategies to study its effect on network activity when assumptions about retrograde messenger's effect or feedback inhibition are either changed or fixed. It is known that synapses may vary in their molecular compositions which lead to demonstrate a different initial release probability (Fernandez-Chacon et al., 2001; Kavalali, 2015). Accordingly, it is important to consider synapses with different initial release probability and its effect on encoding efficiency. This theoretical study assigns a critical role for homeostatic regulation of neurotransmitter release by fast diffused retrograde messenger and Hebbian plasticity in efficient neuronal encoding tasks when synapses are acting as low or band pass filters. Moreover, the model predicts that there are other synaptic mechanisms for neurons with synapses which act as high-pass filters that enable them to encode their inputs with different levels of intensity.

Modeling work in the current study is based on a simple mechanistic implementation of complicated molecular events in which some biophysical properties of agents were simplified. For example, we did not model diffusion of retrograde messenger. Assuming that it acts at very low distances, it can affect partially activated synapses in the network. Our understanding of the cellular mechanism of neurotransmitter release machinery and its inhibition mechanisms, especially the time scale of different underlying mechanisms, may lead to a modification of the dynamics of the model and basic assumptions. However, such modification and improvements need many experiments on the hypothesis and its mechanistic details. Another possibility to improve the model is to consider how structural plasticity (the dynamic connectivity of neurons over time) interact with the change of information flow into a neuron (Lamprecht and LeDoux, 2004; Yin and Yuan, 2015).

The simulations presented in this study predict that impairment in any part of such complex cellular mechanisms may lead to a deficiency in neural encoding of neurons and neural populations. Therefore, it offers an explanation of the role of genetic mutations that may affect biochemical pathways of information processing in neurons which gives rise to synaptic diseases (Chakroborty et al., 2012; Grant, 2012).

Modeling and simulation studies in combination with experiments can help improve our understanding of neural encoding and decoding in different physiological conditions, specifically where cellular parameters like biophysical properties of channels or changing biochemical pathways are not easily assessable to be modified by experimental techniques. The importance of such efforts are better realized when we consider the role of theoretical studies in measuring quantities that are not basically determined by experiments at molecular, cellular or network levels. One of the best examples of such quantities is to measure information transferred by neurons. Until now mainly information theory and some mathematical measures have been used for this purpose. Therefore, applying such measures in simulations especially if they are based on information about cellular architecture and functionality can play a very critical role in designing research plans to explore basic principles of information processing in animal and human the brain.

Acknowledgments

The research is supported by NSF grant number IIS-1302256.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in
CELLULAR NEUROSCIENCE

REVIEW ARTICLE
published: 31 October 2013
doi: 10.3389/fncel.2013.00190

The role of nitric oxide in pre-synaptic plasticity and homeostasis

***Neil Hardingham*[‡], *James Dachtler*[†‡] *and Kevin Fox**

School of Biosciences, Cardiff University, Cardiff, UK

Edited by:
Jaichandar Subramanian, Massachusetts Institute of Technology, USA

Reviewed by:
Joern R. Steinert, University of Leicester, UK
Bernardo Moreno-López, University of Cadiz, Spain

***Correspondence:**
Kevin Fox, School of Biosciences, Cardiff University, Museum Avenue, Cardiff CF10 3AX, UK
e-mail: foxkd@cardiff.ac.uk

[†]***Present address:***
James Dachtler, School of Biomedical Sciences, University of Leeds, West Yorkshire, UK

[‡]*These authors are joint first authors.*

Since the observation that nitric oxide (NO) can act as an intercellular messenger in the brain, the past 25 years have witnessed the steady accumulation of evidence that it acts pre-synaptically at both glutamatergic and GABAergic synapses to alter release-probability in synaptic plasticity. NO does so by acting on the synaptic machinery involved in transmitter release and, in a coordinated fashion, on vesicular recycling mechanisms. In this review, we examine the body of evidence for NO acting as a retrograde factor at synapses, and the evidence from *in vivo* and *in vitro* studies that specifically establish NOS1 (neuronal nitric oxide synthase) as the important isoform of NO synthase in this process. The NOS1 isoform is found at two very different locations and at two different spatial scales both in the cortex and hippocampus. On the one hand it is located diffusely in the cytoplasm of a small population of GABAergic neurons and on the other hand the alpha isoform is located discretely at the post-synaptic density (PSD) in spines of pyramidal cells. The present evidence is that the number of NOS1 molecules that exist at the PSD are so low that a spine can only give rise to modest concentrations of NO and therefore only exert a very local action. The NO receptor guanylate cyclase is located both pre- and post-synaptically and this suggests a role for NO in the coordination of local pre- and post-synaptic function during plasticity at individual synapses. Recent evidence shows that NOS1 is also located post-synaptic to GABAergic synapses and plays a pre-synaptic role in GABAergic plasticity as well as glutamatergic plasticity. Studies on the function of NO in plasticity at the cellular level are corroborated by evidence that NO is also involved in experience-dependent plasticity in the cerebral cortex.

Keywords: LTP (Long Term Potentiation), synaptic plasticity, NOS1, experience-dependent plasticity, guanylate cyclase

INTRODUCTION

Nitric oxide is a ubiquitous signaling molecule in the brain and in other organs of the body. It is involved in an almost bewildering array of functions. Consequently, there have been many reviews over the years that have described its role in retrograde signaling (Brenman and Bredt, 1997), cellular function (Garthwaite, 2008), synaptic plasticity (Holscher, 1997), development (Contestabile, 2000), excitotoxicity (Calabrese et al., 2007), blood flow (Gordon et al., 2007) and mental health (Steinert et al., 2010). However, in this review we focus on the role of NO in synaptic plasticity and specifically its function as a retrograde messenger. It seems fitting to look at the evidence now as it is 25 years since the original discovery that NO (or endothelial derived relaxing factor) might act as an intercellular messenger in the brain (Garthwaite et al., 1988), during which time there has been a steady accumulation of evidence for the role of NO synthase in synaptic plasticity and homeostasis at both excitatory and inhibitory synapses. In the following sections we briefly review the main pathways by which NO acts and the distance over which it acts, before discussing the evidence for its role in synaptic signaling during plasticity and homeostasis.

MOLECULAR PATHWAYS FOR THE ACTION OF NO

Nitric oxide is generated by the enzyme NO synthase (NOS). NOS1 (nNOS or neuronal NOS) is one of three major isoforms of NO synthase, the others being NOS2 (iNOS or inducible NOS) and NOS3 (eNOS or endothelial NOS). Many cell types in the body can express NOS2, including immune response cells (Hickey, 2001), glial cells (Nomura and Kitamura, 1993) and neurons (Corsani et al., 2008). Unlike NOS1 and NOS3 that are expressed constitutively, NOS2 is induced by inflammatory cytokines (Saha and Pahan, 2006). Calcium/calmodulin has such a high affinity for NOS2 that its activity is not modulated by this route, which means that NOS2 activity is under the control of cytokines rather than calcium signaling. Antagonists of NOS2 have been reported to reduce synaptic plasticity and alter both spontaneous and evoked synaptic activity in the cortex (Buskila and Amitai, 2010), although NOS1 may also have been affected at the drug concentrations used in this study.

NOS3 was originally isolated from endothelial cells, and along with other NOS isoforms is present in the tissues of the cardiovascular system (Buchwalow et al., 2002). While early reports suggested NOS3 was located in neurons (Dinerman et al., 1994),

these findings were later rebutted by the same group (Blackshaw et al., 2003). NOS1 knockouts show that NOS1 is the source of 95% of the NO in the cortex (Huang et al., 1993) and plays a major role in synaptic plasticity (see Section NO Controls Pre-Synaptic Function and The Role of NO in Plasticity). However, tonic levels of NO produced by NOS3 may also play a role in the induction of plasticity (Hopper and Garthwaite, 2006).

SOLUBLE GUANYLATE CYCLASE

Soluble guanylyl cyclase (sGC) is the most sensitive receptor for NO, with an EC_{50} in the low nanomolar (nM) range (Roy et al., 2008). A good deal of evidence has been gathered in recent years for its importance in mediating the actions of endogenous NO, predominantly at pre-synaptic locations (Garthwaite, 2010; Neitz et al., 2011; Eguchi et al., 2012; Bartus et al., 2013).

Soluble guanylyl cyclase mediates the production of cGMP from GTP. Three subunits of the protein have been identified, α_1, α_2, and β_1. A functional receptor is a heterodimer consisting of one α and one β subunit. Two isoforms of the receptor exist ($\alpha_1\beta_1$ and $\alpha_2\beta_1$) with a complex regional expression. For example, the $\alpha_1\beta_1$ heteromer is dominant in the caudate-putamen and nucleus accumbens whilst $\alpha_2\beta_1$ is dominant in the hippocampus and olfactory bulb (Gibb and Garthwaite, 2001; Mergia et al., 2003). The $\alpha_2\beta_1$ receptor is present at the highest levels in the brain and the α_2 subunit has been shown to bind to the cell membrane through PSD95 (Russwurm et al., 2001; Mergia et al., 2003), which suggests a post-synaptic localization. The $\alpha_2\beta_1$ isoform can substitute for most functions of the more widely expressed $\alpha_1\beta_1$ isoform despite there being a 90% reduction in sGC in the α_1 KOs (Friebe and Koesling, 2009). However, deletion of the β_1 subunit eliminates expression of any sGC resulting in an 80% infant mortality within 2 days of birth (Friebe and Koesling, 2009). To date, the two α subunit isoforms have only been found to have distinct functions in the induction of LTP in the visual cortex where both isoforms are necessary (Haghikia et al., 2007).

The guanylyl cyclase receptor consists of a haem group of the type that binds O_2 in hemoglobin, but when associated with the receptor protein, it exhibits a substantial preference for NO, allowing detection of NO in the presence of at least 10,000 fold excess of O_2, despite the molecular similarity of the two ligands (Martin et al., 2006).

The mechanism of activation of sGC by NO is complex and involves a conformational change via binding at the haem site, which enables increased conversion of GTP to cGMP (Roy et al., 2008). NO activates guanylyl cyclase within 20 ms and, following removal of NO, activity decays with a half life of 200 ms (Bellamy and Garthwaite, 2001). With formation of cGMP, a bifurcation occurs in the route of action (**Figure 1**); one route is for cGMP to affect cGMP-activated protein kinases (cGKs or PKGs). Multiple substrates for PKG have been identified including PKG activated phosphatases, leading indirectly to altered levels of phosphorylation of effector proteins (Schlossmann and Hofmann, 2005). The second major route of action for cGMP is to bind to agonist or regulatory sites on cyclic nucleotide-gated (CNG) ion channels or hyperpolarization-activated, cyclic nucleotide-modulated (HCN) channels.

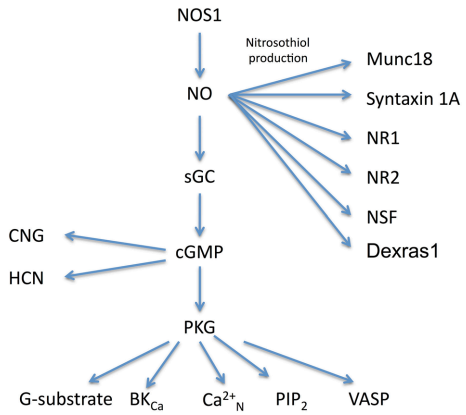

FIGURE 1 | Molecular signaling pathways for NO. The main signaling pathways described in the text for NOS1 are shown together with their effector molecules. NO has three main routes of action via nitrosothiol production, cGMP and PKG. Abbreviations: NOS1, Nitric Oxide Synthase 1; NO, Nitric Oxide; sGC, soluble guanylate cyclase; PKG, protein kinase G; G-substrate, a phosphatase inhibitor; BK_{Ca}, large calcium sensitive potassium channel; Ca2+N, N-type calcium channel; PIP2, phosphotidylinositol 4,5 biphosphate; VASP, vasodilator stimulated phosphoprotien; CNG, cyclic nucleotide gated channel; HCN, hyperpolarization-activated, cyclic nucleotide-modulated channel; Munc18, also known as Sec-1, is a pre-synaptic SNARE associated protein; syntaxin 1A, part of the SNARE complex; NR1, NMDA receptor subunit 1; NR2, NMDA receptor subunit 2; NSF, N-ethylmaleimide sensitive fusion protein. There is evidence for nitrosothiol production in NSF, NR1, and NR2 *in vivo*, but endogenous production of nitrosothiol groups in syntaxin requires confirmation.

PRODUCTION OF NITROSOTHIOL GROUPS

There are a number of cases where NO signaling in the brain is transduced in a cGMP independent manner. The thiol side chains of cysteine residues in proteins can be modified by the addition of an NO group and this outcome could occur by two known routes: the thiol group can be oxidized to a thyl followed by addition of NO, which is known as oxidative nitrosylation, or NO can react with O_2 to produce N_2O_3 which then interacts with the thiol group to produce nitrosothiol, and this process is known as nitrosation (Heinrich et al., 2013). At present, the endogenous route for nitrosothiol production is not known.

A number of pre-synaptic proteins have been identified as potential targets for nitrosothiol production and therefore as a mechanism for mediating alterations in pre-synaptic strength (**Figure 1**). The t-snare protein synapsin has been identified as a target for nitrosothiol production in pancreatic cells (Wiseman et al., 2011) and syntaxin 1a and n-sec1 (also known as Munc18) have been shown to be a target for nitrosothiol production in neurons (Meffert et al., 1996; Prior and Clague, 2000; Palmer et al., 2008). A small GTPase known as Dexras1 (which can be induced by dexamethasone) is held in close proximity to NOS1 by CAPON (Jaffrey et al., 1998) and can be modified by production of nitrosothiol (Fang et al., 2000).

Nitrosothiol production requires much higher concentrations of NO than activation of sGC and proceeds with slower kinetics. For example, nitrosothiol production in syntaxin 1A occurs with an IC_{50} of 1.1 μM NO (Palmer et al., 2008) compared with

the nM range of detection for sGC (Roy et al., 2008). It has been estimated that an NO concentration of 200 μM would require 2 min to produce nitrosothiol groups in half the substrate (Ahern et al., 2002). The high concentrations and slow reaction kinetics of nitrosothiol production raise the question of whether it can occur naturally. Most of the experiments conducted on production of nitrosothiol groups in various proteins use NO donors at quite high levels [for example 100–1000 μM for nitrosothiol production in SNAP25 (Di Stasi et al., 2002)]. However, a technique for detecting nitrosothiol groups in proteins known as the biotin switch method has been used to demonstrate the existence of endogenous nitrosothiol groups *in vivo* by comparing results in wild-type mice with NOS1 knockout mice (Jaffrey et al., 2001). The synaptic proteins that appear to have endogenous nitrosothiol groups using this method include NR1, NR2A (Jaffrey et al., 2001), and NSF (Huang et al., 2005).

It may not be coincidental that some of the molecules shown to have nitrosothiol groups *in vivo* are held in close proximity to NOS1 and thereby experience the higher source concentrations of NO. The NMDA receptor is local to NOS1 by virtue of them both binding to PSD95 and dexras1 is close to NOS1 because both bind to CAPON (Fang et al., 2000). It may also be relevant that nitrosothiol groups occur on molecules that tend to lie close to lipid membranes, in this case synaptic membranes. It has been suggested that the kinetics of the reaction between NO and O_2 to produce N_2O_3 could be increased by NO and O_2 becoming concentrated in lipid membranes (Heinrich et al., 2013). However, once again it should be emphasized that the endogenous routes for generating nitrosothiol groups on proteins are not known at present.

THE CELLULAR LOCATION OF NOS1

NOS1 is composed of several splice variants. The long form of NOS is αNOS1 which contains a PDZ binding domain that enables it to bind to the PDZ2 domain of PSD95 (Brenman et al., 1996; Eliasson et al., 1997) localizing NOS1 to the post-synaptic density (see Doucet et al., 2012). There are also shorter splice variants of NOS1 lacking the PDZ domain known as βNOS1 and γNOS1. While the latter is not expressed very highly in the brain, βNOS1 is expressed quite highly in the ventral cochlear nuclei, the striatum and the lateral tegmental nuclei (Eliasson et al., 1997). In the cortex and hippocampus, the current evidence suggests that NOS1 is located in two very different neuronal compartments in two different cell types. On the one hand, NOS1 is located in the cytoplasm of a small subpopulation of GABAergic cells in the cortex and hippocampus and on the other, it is located in a far larger population of excitatory neurons, but highly restricted to the spine head. The ease with which NOS1 can be detected at the two locations depends on the techniques used as described below.

LIGHT MICROSCOPY

The light microscopy (LM) level is sufficient to demonstrate the presence of cytoplasmic NOS1 (Eliasson et al., 1997; Blackshaw et al., 2003; Kubota et al., 2011). LM antibody studies have shown that the strongest NOS1 staining in the neocortex and hippocampus occurs in a small subpopulation of GABAergic neurons (Wendland et al., 1994; Aoki et al., 1997; Blackshaw et al., 2003) that co-express Somatostatin, Neuropeptide Y and the Substance P receptor (Kubota et al., 2011). The $NOS1^+$ GABAergic neurons contain both αNOS1 and βNOS1. A significant component of the cytoplasmic staining is attributable to βNOS1 as it persists in αNOS1 knockouts (Eliasson et al., 1997). Weaker labeling of the cortical neuropil is also consistently reported in the same papers. Recent studies using targeted knockin of cre-recombinase into the NOS1 gene and subsequent crosses to GFP reporter lines clearly show two populations of $NOS1^+$ GABAergic cells, one of neurogliaform morphology (type II) and the other characterized by long range axonal projections (type I) (Taniguchi et al., 2011). Again the neuropil can be seen throughout the cortical layers including clear axonal labeling (**Figure 2**). Pyramidal cell labeling is not seen in these cre lines, however, possibly due to the technique only showing high levels of NOS1 expression (Josh Huang personal communication). Weak labeling of CA1 pyramidal cells can be seen using NOS1 antibodies with the right fixative conditions (Burette et al., 2002; Blackshaw et al., 2003) and colocalization of NMDA, PSD95, and NOS1 shows that some of the punctate labeling seen with LM is due to NOS1 in spines (Burette et al., 2002).

ELECTRON MICROSCOPY

Using electron microscopy (EM), much of the neuropil labeling present in LM studies can be seen to reside in the axons of NOS^+ GABAergic neurons (Aoki et al., 1997). However, EM studies reveal a further component of the neuropil labeling to be due to the very precise and restricted localization of NOS1 in spines, spine heads, and occasionally the plasma membrane of dendrites

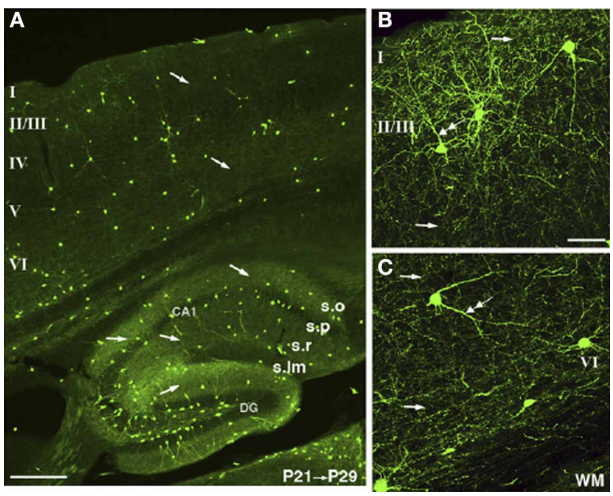

FIGURE 2 | NOS1 positive cells at the LM level in the Cortex and Hippocampus. Cells expressing TdTomato fluoresce in nNOS positive cells in an nNOS-CreER;Ai9 mouse. The TdTomato is rendered green in the images. **(A)** The nNOS positive cells make up a small population scattered in cortex and hippocampus. **(B)** A dense and diffuse plexus of neuropil can be seen throughout layer II/III and **(C)** throughout deeper layers of the cortex. Single arrows indicate axons and double arrows dendrites. Adapted from Taniguchi et al. (2011) with kind permission of the authors and Cell press.

(Aoki et al., 1997, 1998). The NOS1 visible in the heads of spines in the visual cortex and in some cases at the base of spines accounts for 30–75% of the punctate labeling in cortical electron micrographs (**Figure 3**). Although the NOS1$^+$ GABAergic neurons are sparsely spiny and could theoretically account for some of the NOS1 spine labeling, the extent of the spine labeling seen in EM is too great to be due purely to GABAergic cells (Cheri Aoki personal communication); therefore a considerable amount of spine labeling must be attributable to excitatory pyramidal cells. Furthermore, the NOS1 labeling in spines is quite distinctive in that the labeled spines are joined to dendrites that do not contain NOS1 labeling (**Figure 3**); if these spines were located on GABAergic cells, the cytoplasm would be labeled as well. EM studies of cortical synapses also show that the gold particle distribution associated with NOS1 labeling is coextensive with that for PSD95 relative to the plasma membrane (Valtschanoff and Weinberg, 2001). Similarly, in the hippocampus, EM studies show that NOS1 is located in dendritic spines on pyramidal cells (Burette et al., 2002). The NO receptor sGC is found pre-synaptic and within 50–150 nm of the NOS (**Figure 4**). In conclusion, pyramidal cells in the neocortex and hippocampus contain NOS1 that is highly localized to the spine head, spine neck, or plasma membrane of the dendrites and is closely apposed to pre-synaptic sGC.

MOLECULAR AND FUNCTIONAL METHODS

The reason why NOS1 is localized to the spine head is due to the nature of the alpha sub-isoform of NOS1 which contains a PDZ binding domain that enables it to bind to the PDZ2 domain of PSD95 (Brenman et al., 1996; Eliasson et al., 1997). Using proteomic analysis of molecules associated with the NMDA receptor, it has been shown that NOS1 is part of the NMDA signaling complex (Husi et al., 2000). The authors used a combination of immunoaffinity chromatography, immunoprecipitation with an antibody directed against the NR1 subunit, and peptide affinity based on the structure of the NR2B subunit C terminus that binds to the NMDAR-binding protein PSD-95. The structure and binding partners of NOS1 and PSD95 are reviewed in (Zhou and Zhu, 2009) and (Doucet et al., 2012).

Functional assays also demonstrate the synaptic location of NOS1. The functional consequences of disrupting the interaction between NOS1 and PSD95 has been studied by expressing decoy proteins that code for amino acids constituting the PDZ binding domain of αNOS1. For example, glutamate induced activation of p38 normally leads to excitotoxic cell death, but this process can be prevented by expression of the first 300 amino acids of NOS1 ($NOS1_{1-300}$) (Cao et al., 2005). Similarly, cerebral ischemia induced by cerebral artery occlusion leads to cortical damage which can be reduced by

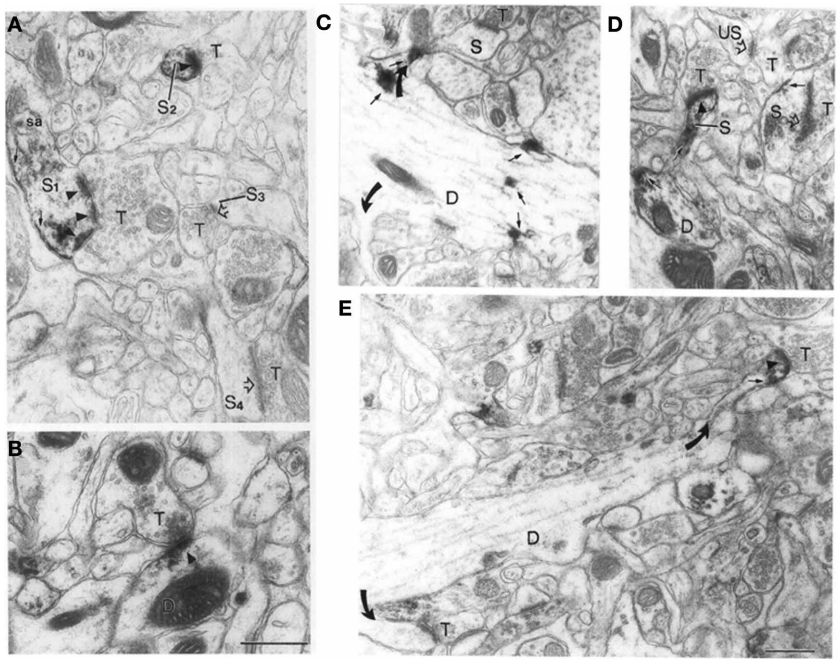

FIGURE 3 | NOS1 positive spines at the EM level in the Visual Cortex. (A) Large dendritic spine (S1) with a perforated PSD showing NOS1-immunoreactivity (arrowheads). NOS1 immunoreactivity is also present along the plasma membrane (small arrow) and near the spine apparatus (sa). A second small spine (S2) shows NOS1 immunoreactivity along the plasma membrane and over the PSD. Not all spines are labeled (S3 and S4). T represents unlabeled pre-synaptic terminals. Open arrows mark unlabeled PSDs. **(B)** Axodendritic synapse showing NOS1 labeling of a PSD (arrowhead). **(C)** NOS1 labeling occurs at the spine base (upper curved arrow) and dendritic shaft (small arrows). Lower curved arrow points to an unlabeled spine. S is a spine head and D is a dendritic shaft where limited NOS1 labeling occurs along the plasma membrane. **(D)** NOS1 immunoreactivity over the spine neck (S), plasma membrane forming the spine head (small arrow) and the PSD (filled arrowhead). US marks an unlabeled spine and open arrowheads also mark unlabeled spines and T is the pre-synaptic terminal. **(E)** NOS1 immunoreactivity only in the spine head. Note that in all these cases there is no labeling of the dendritic cytoplasm. Calibration bar = 500 nm. Adapted from Aoki et al. (1998) with kind permission of the author and Elsevier press.

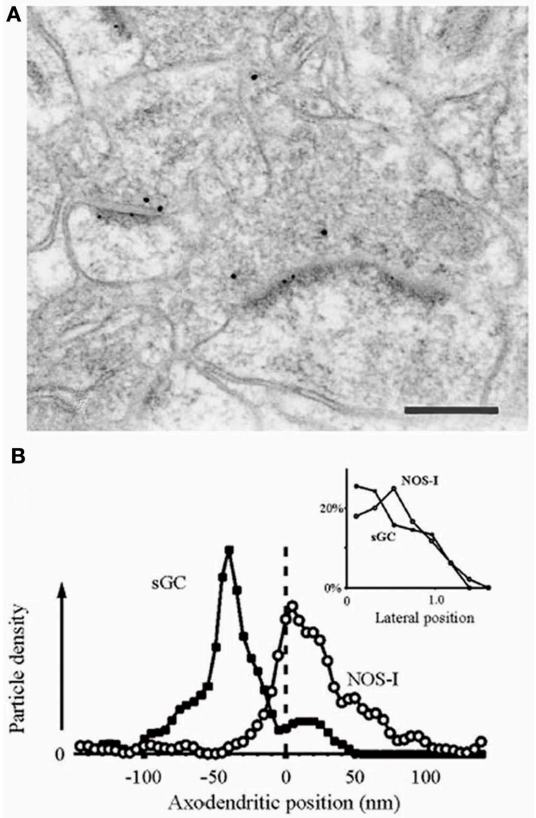

FIGURE 4 | NOS1 positive spines and sGC positive terminals in the hippocampus. (A) Positions of gold particles identifying NOSI and sGC located within 150 nm of the post-synaptic membrane. Inset, labeling close to the plasma membrane is concentrated at the synaptic specialization for both antigens. **(B)** Double immunogold labeling showing that NOS1-positive PSDs lie post-synaptic to sGC-positive axon terminals. Small dots are 5 nm gold particles labeling NOS1. Large dots are 10 nm gold particles labeling sGCβ. Scale bar is 200 nm. Adapted from Burette et al. (2002) with kind permission of the authors and the Society for Neuroscience.

$NOS1_{1–133}$ (Zhou et al., 2010) and pTAT-PDZ1-2 (Aarts et al., 2002). Thermal hyperalgesia and chronic mechanical allodynia can be inhibited by intrathecal application of IC8731 or tat-NOS1 (NOS1 1-299) (Florio et al., 2009). These molecules do not act to reduce the enzymatic activity of NOS1, but rather to decrease the coupling between NOS1 and NMDA receptors by disrupting the ability of NOS1 to bind to PSD95 (Florio et al., 2009).

Finally, studies on synaptic plasticity (as described in Section The Role of NO in Plasticity), show that the pre-synaptic NO-dependent component of LTP can be prevented by post-synaptic application of NOS antagonists to layer 2/3 pyramidal cells in the somatosensory cortex (Hardingham and Fox, 2006). Similar results have been demonstrated for layer 5 cortical cells (Sjostrom et al., 2007). This implies that the NO synthase exists in pyramidal cells in the cortex.

In conclusion, LM studies are able to demonstrate the presence of NOS1 in the NOS^+ GABAergic cells of the neocortex and hippocampus but LM is at the limit for demonstrating its presence in pyramidal cells, while EM, proteomic, and functional analysis are sensitive enough to demonstrate the presence of NOS1 at spines of pyramidal cells.

THE PHYSIOLOGICAL CONCENTRATION OF NO AND ITS DISTANCE OF ACTION

A theoretical consideration of the rate of production of NO at an individual synapse suggests that NO has a source concentration in the low nanomolar range. Working forward from a knowledge of the rate of NO production per NOS molecule *in vitro* of 20 per second (Santolini et al., 2001) and using an estimate of the number of NMDA receptors and therefore NOS molecules present at a single post-synaptic density, the concentration in the immediate vicinity of the NOS molecule can be estimated at 2.5 nM, falling 10 fold within approximately 700 nm (Hall and Garthwaite, 2009). Working backwards from a measure of NO concentration generated in a cerebellar slice stimulated with NMDA gives a similar rate of production of NO per NOS molecule (10 per second) and a source concentration at the synapse of approximately 0.01–0.1 nM (Wood et al., 2011). A number of studies have reported that NO is produced in the brain in the picomolar range (Wakatsuki et al., 1998; Sato et al., 2006; Wood et al., 2011) and several other labs in the low nM range (<10 nM) (Shibuki and Kimura, 1997; Kimura et al., 1998; Wu et al., 2001, 2002; Sammut et al., 2006, 2007a,b; Ondracek et al., 2008; Sammut and West, 2008).

If the concentration of NO produced at a synapse is in the pM to low nM range, then the rate of inactivation of NO with distance in the brain implies that it can only act over a relatively short range. The most sensitive target for NO is soluble guanylate cyclase (sGC), which can respond to as little as 1 pM NO (Batchelor et al., 2010). The EC_{50} of sGC to NO is thought to be in the low nanomolar range at 1.7 nM (Griffiths et al., 2003). Physiological concentrations of ATP (1 mM) and GTP (0.1 mM), which antagonistically decrease and increase the sensitivity of sGC to NO, respectively, elevate the EC_{50} to 3.4 nM (Roy et al., 2008). Taking into account both the likely concentration of NO at the synapses and the sensitivity of sGC suggests that NO is only likely to act over distances of less than 1 micron.

The lower estimate of NO evolution in the picomolar range would sit on the non-linear cusp of the NO/sGC binding curve (Roy et al., 2008). This raises the interesting possibility that a tonic level of NO production could interact with the NMDA receptor activated NO concentration to boost its effect on sGC. For example, a tonic level of 250 pM NO would move the operating point of the synapse onto the linear part of the NO/sGC curve [see Figure 7B of Roy et al. (2008)]. There is evidence for a tonic level of NO production in the brain originating from both NOS3 and NOS1 (Hopper and Garthwaite, 2006; Dachtler et al., 2011). Furthermore, tonic levels of NO have been found to influence the magnitude of LTP, giving further credence to this notion. NO donors can be shown to facilitate both post-synaptic potentials and LTP (Bohme et al., 1991; Malen and Chapman, 1997; Hardingham and Fox, 2006). The higher estimate of NO release in the nM range would not require background levels of NO to move sGC on to the linear part of its response curve. With either mode of action, NO would only be able to act over a distance of

less than about 1 micron, effectively making it a synapse specific signal.

The view of NO as a synapse specific signal does not fit with the notion of NO as a volume transmitter. Nevertheless, there is evidence for NO acting as a volume transmitter in the Calyx of Held (Steinert et al., 2008). Theoretically, all that would be required for higher concentrations of NO would be higher concentrations of the enzyme NOS. It is conceivable that the GABAergic inhibitory cells that express NOS1 at much higher levels than excitatory cells (**Figure 2**) throughout their cytoplasm could provide such a source. The $NOS1^+$ GABAergic cells produce a plexus of fine NOS positive fibers that ramify throughout the cortex and hippocampus, which could aid spatial summation of NO levels. However, little is known of NO release from this small subpopulation of cells at present.

NO CONTROLS PRE-SYNAPTIC FUNCTION

The past two decades have seen a steady but decisive accumulation of evidence showing not only that NO acts pre-synaptically on neurotransmitter release, but how it does so (Feil and Kleppisch, 2008). **Table 1** is a compilation of papers showing some of the evidence for NO's pre-synaptic action, its retrograde route from post- to pre-synaptic site and its pre-synaptic action in plasticity.

Much of the detailed evidence for NO's role in transmitter release comes from studies on the glutamatergic system, but a body of work implicates NO in regulating transmitter release from GABAergic (Kawaguchi et al., 1997; Li et al., 2002; Moreno-Lopez et al., 2002; Wall, 2003; Szabadits et al., 2007; Yang et al., 2007; Bright and Brickley, 2008; Xue et al., 2011; Lange et al., 2012) dopaminergic (West et al., 2002) and noradrenergic synapses (Montague et al., 1994; Kodama and Koyama, 2006).

A number of the studies providing evidence for the retrograde action of NO have come from cell cultures. Cell culture preparations have a number of technical advantages that allow the retrograde action of NO to be demonstrated (**Table 1**). However, since cells in culture are immature, it raises the question of whether NO acts the same way in more mature cells. Nevertheless, a number of studies made on mature neurons in intact slices of hippocampus (O'Dell et al., 1991; Schuman and Madison, 1991), amygdala (Lange et al., 2012), neocortex (Hardingham and Fox, 2006; Sjostrom et al., 2007), the medial nucleus of the trapezoid body (Steinert et al., 2008; Eguchi et al., 2012), cerebellum (Qiu and Knopfel, 2007), and the ventral lateral medulla (Huang et al., 2003), lead to similar conclusions about the action of NO in mature cells, suggesting that NO retains its retrograde pre-synaptic action into adulthood.

In the following sections we briefly review the findings for NO's effects on four aspects of pre-synaptic function; actions at the active zone, on vesicle recycling, effects on the readily releasable pool and actions on pre-synaptic growth. When viewed in combination, these studies suggest that NO may regulate pre-synaptic release by acting in a coordinated and synergistic manner on several aspects of pre-synaptic release (**Figure 5**).

EFFECTS ON THE ACTIVE ZONE AND TRANSMITTER RELEASE

Nitric oxide can affect transmitter release by nitrosothiol generation in a number of constituents of the active zone (**Figure 5B**). For example, nitrosothiol production in syntaxin at Cys(145) has a facilitatory effect on release because it prevents munc18 (also known as n-sec1) from binding to the closed conformation of syntaxin 1a. This allows syntaxin1a to unfold and bind to both VAMP on the vesicle and SNAP25 at the release site, which in turn enables the vesicle to dock to the membrane (Meffert et al., 1996; Palmer et al., 2008). SNAP25 can itself have nitrosothiol groups generated by NO, which may further enhance release (Di Stasi et al., 2002). However, it is not clear at present whether the concentrations of NO necessary for production of nitrosothiol groups are realized at the synapse (see Sections The Cellular Location of NOS1 and The Physiological Concentration of NO and Its Distance of Action).

EFFECTS ON ION CHANNELS

Voltage gated ion channels that reside in the pre-synaptic terminal and affect transmitter release have been shown to be NO sensitive. In the peptidergic synapse of the pituitary nerve, NO can increase pre-synaptic release by enhancing the activity of large conductance Ca^{2+} activated K^+ channels (BK). PKG only activates BK at depolarized potentials, which means that the action potential after-hyperpolarization becomes larger without affecting the spike threshold. Consequently, during prolonged trains of action potentials, the enhanced hyperpolarization provided by BK channels accelerates Na^+ channel recovery (Klyachko et al., 2001). It can be demonstrated that cytosolic calcium almost doubles in the presence of exogenous cGMP. A possible physiological role for this action is suggested by showing that the action potential success rate during a 25 Hz stimulus train is almost twice as great in the control condition when compared to that in the presence of the NO synthase inhibitor 7-NI or the sGC inhibitor ODQ (Klyachko et al., 2001). In the brainstem, synaptic potentials generated by glutamatergic synapses in the ventrolateral medulla can be enhanced by application of the NOS substrate L-arginine (200 uM) (Huang et al., 2003). This effect can be shown to be due to NO acting via a cGMP/protein kinase G-dependent pathway on N-type calcium channels (Huang et al., 2003). It is not known at present whether BK or N-type calcium channels are affected by NO in the cortex or hippocampus.

One other means by which NO may affect transmitter release in some types of neuron is by stimulating the production of cGMP, which directly gates cyclic nucleotide gated channels (Neitz et al., 2011). Cyclic nucleotide gated (CNG) channels are well known for their function in transmitter release in some classes of cell, for example photoreceptors (Rieke and Schwartz, 1994) and olfactory epithelial cells (Leinders-Zufall et al., 2007). However, the distribution of CNG channels is more widespread and roughly mirrors the distribution of the NO/cGMP system (Kingston et al., 1999). For example, CNG channels are present in the rat hippocampus (Kingston et al., 1999) and may be involved in the induction of theta burst LTP in mouse hippocampus (Parent et al., 1998). While native heteromeric CNG channels formed by alpha and beta subunits are gated by cGMP, homomeric channels comprising just the beta subunit are directly activated by NO (Broillet and Firestein, 1997), raising the possibility that NO might act on native CNG channels

Table 1 | Evidence that nitric oxide influences presynaptic function.

References	Title	Presynaptic action?	Retrograde messenger?	Effect on plasticity?	Transmitter	Structure (preparation)
Arancio et al., 1996a	Nitric oxide acts directly in the presynaptic neuron to produce long-term potentiation in cultured hippocampal neurons	✓	✓	✓	Glutamate	Hippocampus (cell culture)
Lange et al., 2012	Heterosynaptic long-term potentiation at interneuron-principal neuron synapses in the amygdala requires nitric oxide signaling	✓	✓	✓	GABA	Amygdala (slices)
O'Dell et al., 1991	Tests of the roles of two diffusible substances in long-term potentiation: evidence for nitric oxide as a possible early retrograde messenger	✓	✓	✓	Glutamate	Hippocampus (slices)
Sjostrom et al., 2007	Multiple forms of long-term plasticity at unitary neocortical layer 5 synapses	✓	✓	✓	Glutamate	Visual cortex (slices)
Hardingham and Fox, 2006	The role of nitric oxide and GluR1 in presynaptic and postsynaptic components of neocortical potentiation	✓	✓	✓	Glutamate	Barrel cortex (slices)
Schuman and Madison, 1991	A requirement for the intercellular messenger nitric oxide in long-term potentiation	✓	✓	✓	Glutamate	Hippocampus (slices)
Volgushev et al., 2000	Retrograde signaling with nitric oxide at neocortical synapses	✓	✓	✓	Glutamate	Visual cortex (slices)
Montague et al., 1994	Role of NO production in NMDA receptor-mediated neuro-transmitter release in cerebral cortex	✓	✓		Glutamate	Neocortex (synaptosomes)
Micheva et al., 2003	Retrograde regulation of synaptic vesicle endocytosis and recycling	✓	✓		Glutamate	Hippocampus (cell culture)
Eguchi et al., 2012	Maturation of a PKG-dependent retrograde mechanism for exoendocytic coupling of synaptic vesicles	✓	✓		Glutamate	MNTB/Caylx of Held (slices)
Lindskog et al., 2010	Postsynaptic GluA1 enables acute retrograde enhancement of presynaptic function to coordinate adaptation to synaptic inactivity	✓		✓	Glutamate	Hippocampus (cell culture)

(Continued)

Table 1 | Continued

Qiu and Knopfel, 2007	An NMDA receptor/nitric oxide cascade in presynaptic parallel fiber-Purkinje neuron long-term potentiation	✓	✓	Glutamate	Cerebellum (slices)
Johnstone and Raymond, 2011	A protein synthesis and nitric oxide-dependent presynaptic enhancement in persistent forms of long-term potentiation	✓	✓	Glutamate	Hippocampus (slices)
Stanton et al., 2005	Imaging LTP of presynaptic release of FM1-43 from the rapidly recycling vesicle pool of Schaffer collateral-CA1 synapses in rat hippocampal slices	✓	✓	Glutamate	Hippocampus (slices)
Wang et al., 2005	Presynaptic and postsynaptic roles of NO, cGK, and RhoA in long-lasting potentiation and aggregation of synaptic proteins	✓	✓	Glutamate	Hippocampus (cell culture)
Arancio et al., 2001	Presynaptic role of cGMP-dependent protein kinase during long-lasting potentiation	✓	✓	Glutamate	Hippocampus (cell culture)
Huang et al., 2003	cGMP/protein kinase G-dependent potentiation of glutamatergic transmission induced by nitric oxide in immature rat rostral ventrolateral medulla neurons *in vitro*	✓		Glutamate	Ventrolateral medulla (slices)
Ratnayaka et al., 2012	Recruitment of resting vesicles into recycling pools supports NMDA receptor-dependent synaptic potentiation in cultured hippocampal neurons	✓		Glutamate	Hippocampus (cell culture)
Neitz et al., 2011	Presynaptic nitric oxide/cGMP facilitates glutamate release via hyperpolarization-activated cyclic nucleotide-gated channels in the hippocampus	✓		Glutamate	Hippocampus (slices)

by two routes. Finally, at the glutamatergic neuromuscular junction in Drosophila, calcium independent vesicular release can result from cGMP triggered by NO (Wildemann and Bicker, 1999), although the exact downstream processes by which this occurs are not known. Calcium independent vesicular release can also be observed in hippocampal synaptosomes (Meffert et al., 1994).

EFFECTS ON VESICLE RECYCLING

In order to sustain synaptic release over a period of time, the rate of vesicle recycling needs to at least equal the rate of vesicle exocytosis. This issue is particularly problematic for synapses that release transmitter at high rates, such as those located at the Calyx of Held that terminate on neurons of the medial nucleus of the trapezoid body (MNTB). Part of the solution to

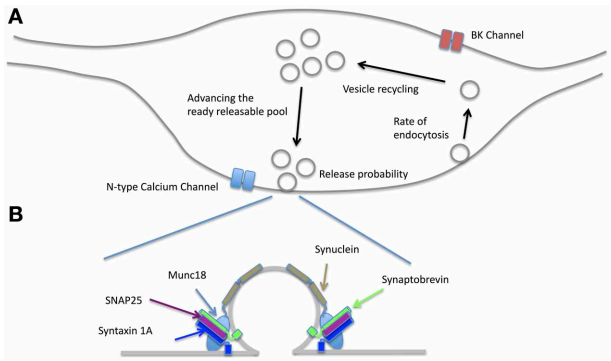

FIGURE 5 | Effects of NO on the pre-synaptic terminal. (A) NO affects release-probability most likely through a combination of effects including enhancing N-type calcium channel conductance via PKG, increasing the rate of endocytosis and vesicle recycling as well as altering the balance of the readily releasable pool via PKG and PIP_2. By acting on BK channels, the probability of action potential failures during moderate spike rates is reduced (see text for the related references). **(B)** There is evidence that many of the SNARE proteins are affected by NO. Syntaxin 1A and SNAP25 can have nitrosothiol groups added although whether this happens at physiological NO concentrations is yet to be established. NO also creates nitrosothiol groups on Munc18 and thereby disinhibits syntaxin from forming the SNARE complex. Alpha Synuclein is also affected by NO signaling. Synaptobrevin is not known to be affected by NO.

this problem at the Calyx is provided by linking vesicle recycling to retrograde release of post-synaptic NO. Activation of the post synaptic MNTB neurons is related to the level of NO production (Steinert et al., 2008), which then drives the level of pre-synaptic cGMP production and hence the level of PKG activity (Eguchi et al., 2012). Finally, activation of PKG up-regulates PIP2, which increases the rate of endocytosis (Eguchi et al., 2012). This homeostatic mechanism therefore links pre-synaptic rate of release (which is sensed by post-synaptic NOS1) to the rate of pre-synaptic vesicle recycling (**Figure 5**). Regulation of the recycling rate has also been demonstrated in the hippocampus, where a very similar retrograde NO—pre-synaptic cGMP/PIP2 cascade regulates the rate of endocytosis and recycling (Micheva et al., 2003).

EFFECTS ON AVAILABILITY AND SIZE OF THE READILY RELEASABLE POOL (RRP)

Studies aimed at investigating the nature of synaptic plasticity have shown that LTP is accompanied by an increase (and LTD a decrease) in the rate of vesicular release from the readily releasable pool (RRP). The LTP process is NMDA receptor-, tyrosine kinase- and NO-dependent while the LTD process is NMDA-, NO- and PKG-dependent (Stanton et al., 2003, 2005). Studies have shown that the size of the RRP can be modulated by NO (**Figure 5**). For example, in the case of LTP, NMDA receptor activation leads to NO and calcineurin activation, which combine to increase the proportion of vesicles available for release (i.e., increase the RRP) (Ratnayaka et al., 2012). Once again this can be seen as a homeostatic response to an increase in release probability brought about by the process of LTP itself. The two processes are coordinated because NO is involved both in increasing transmitter release and increasing the size of the readily releasable pool.

EFFECTS ON GROWTH OF PRE-SYNAPTIC TERMINALS

Nitric oxide also affects the growth and formation of new pre-synaptic terminals and can lead to the formation of multi-innervated spines. Long lasting potentiation leads to an increase in pre- and post-synaptic proteins in hippocampal cell cultures. GluA1 subunits of the AMPA receptor increase post-synaptically and synaptophysin increases pre-synaptically (Antonova et al., 2001). Furthermore, the two synaptic markers co-localize at higher frequency following long lasting potentiation, indicating that new synapses are formed. It has been shown that NMDA receptors, NO and actin are required for the pre-synaptic changes. NO acts via PKG to phosphorylate VASP (which acts on actin) and also via cGMP to act in parallel and downstream of RhoGTPase (Wang et al., 2005).

Further evidence for the role of NO in pre-synaptic growth comes from studies manipulating the PDZ2 domain of PSD95 (which is the PDZ domain that binds NOS1). Up-regulation of PSD95 in cultured hippocampal neurons or treatment with an NO donor leads to the formation of multi-innervated spines (MIS). However, if the PDZ2 domain on PSD95 is deleted, thereby dissociating NOS1 from PSD95, multi-innervated spines fail to form (Nikonenko et al., 2008). Similarly, down regulating NOS1 expression with iRNA also prevents MIS from forming (Nikonenko et al., 2008). Finally, increasing SAP97 expression leads to an increase in PSD95 and again an increase in MIS (Poglia et al., 2011). This effect is blocked by NOS antagonists (Poglia et al., 2011).

In conclusion, the studies cited above show that NO is not only involved in the relatively short term changes involved in transmitter release, such as recycling rates and availability of vesicles, but also, in the long-term, in increasing the availability of transmitter by formation of new pre-synaptic terminals, which results in dendritic spines receiving extra pre-synaptic terminals. Such processes could find application in synaptic plasticity. In the following section we review the function of NO in plasticity and examine to what extent the retrograde route of action is involved.

THE ROLE OF NO IN PLASTICITY

NO-DEPENDENT PRE-SYNAPTIC PLASTICITY

Some of the earliest studies on the role of NO in synaptic plasticity indicated that it might act at a pre-synaptic locus (O'Dell et al., 1991). Exogenous NO applied to neurons in a hippocampal slice increased spontaneous mini EPSCs and hemoglobin acting as an extracellular scavenger for NO was found to prevent LTP (O'Dell et al., 1991). Indeed, initial studies on the mechanisms of LTP itself provided evidence for a pre-synaptic locus of LTP expression (Malinow and Tsien, 1990). In a series of experiments on cultured hippocampal neurons, Arancio and colleagues showed that cGMP (the downstream effector of NO) needs to be pre-synaptic and NOS post-synaptic to produce plasticity. First, cGMP causes an increase in EPSC amplitude when injected into the pre-synaptic but not the post-synaptic cell (Arancio et al., 1995). Second, application of a PKG antagonist

peptide blocks tetanus induced LTP when injected into the pre-synaptic but not the post-synaptic neuron (Arancio et al., 2001). Third, application of a cGMP analogue increases miniature EPSC frequency and this effect is blocked by a post-synaptically but not pre-synaptically injected NOS inhibitor (Arancio et al., 1996a). Forth, a pre-synaptic injection of an NO scavenger also abolishes LTP (Arancio et al., 1996b). More recent work employing fluorescent markers of pre-synaptic function have visualized the pre-synaptic effect of NO in potentiation. Fluorescence imaging of FM-styryl dyes and synaptophysinI-pHluorin has shown that increases to the pre-synaptic recycling pool fraction following synaptic strengthening are dependent upon both NMDA receptor activation and NO release (Ratnayaka et al., 2012).

THE EFFECT OF INITIAL RELEASE-PROBABILITY ON THE LOCUS OF PLASTICITY

Early studies on hippocampal plasticity showed that the initial release-probability of the synapse influences whether a pre- or post-synaptic change occurs following LTP (Larkman et al., 1992). If the release-probability of the synapse is low initially then pre-synaptic plasticity occurs, whereas if the pre-synaptic release-probability is high, then a post-synaptic change occurs (Larkman et al., 1992). A similar principal operates at neocortical synapses. In visual cortex, the initial release-probability of the synapse, as judged by the paired pulse ratio (PPR), is predictive of whether NO-dependent potentiation occurs. Using a purely post-synaptic tetanus (without intentionally eliciting action potentials in the pre-synaptic terminals), potentiation occurs in synapses with a low initial PPR and depression or no change occurs in synapses with a high initial PPR (Volgushev et al., 2000). The same conclusion is arrived at if a paired pre- and post-spike conditioning protocol is used. Low release-probability synapses potentiate via changes in release-probability and high release-probability synapses depress (Hardingham et al., 2007). This normalization process causes the population of connections to adopt a more homogenous set of release probabilities after the protocol. These studies lead to two important conclusions; first, the direction of pre-synaptic plasticity acts in a homeostatic manner to move release-probability to an intermediate value and second, that pre-synaptic plasticity occurs provided that there is sufficient dynamic range for it to occur. There is less scope for increasing release-probability at a high release-probability synapse than at a low release-probability synapse. Potentially, a high release-probability synapse could show pre-synaptic potentiation by growth and/or production of MIS, which can occur and is NO-dependent (section Effects on Growth of Pre-Synaptic Terminals), but structural changes are unlikely within the timescale of an LTP experiment.

Since the initial release-probability of the synapse is an important determinant of the locus of plasticity and in which direction it operates, factors that control initial release-probability will determine the level and form of pre-synaptic plasticity. Adenosine is known to affect release-probability (Prince and Stevens, 1992) and a recent study in layer 5 of the somatosensory cortex has shown that adenosine levels are low early in development (P11-P22) and higher in older animals (P28-32) (Kerr et al., 2013). This maturational change means that adenosine reduces release-probability in older animals, thereby increasing the dynamic range for pre-synaptic potentiation. Some mutant mice strains have unusually low initial release-probability synapses that can provide an increased dynamic range for LTP. For example, H-Ras^{G12V} mice have low release-probability synapses in the visual cortex, as judged by short-term dynamics and mini EPSP frequency, and consequently enhanced LTP with an increased pre-synaptic component (Kaneko et al., 2010).

PRE- AND POST-SYNAPTIC COMPONENTS OF PLASTICITY

Early studies on the role of NO in LTP using NO antagonists often found an absolute requirement for NO (Bohme et al., 1991; O'Dell et al., 1991; Schuman and Madison, 1991; Haley et al., 1993; Doyle et al., 1996; Malen and Chapman, 1997), whereas more recent studies have found LTP to be reduced rather than abolished in the absence of NO, both in the hippocampus (Phillips et al., 2008) and in the neocortex (Hardingham and Fox, 2006).

In the neocortex, LTP occurs as a mixture of pre- and post-synaptic changes, but the two components can be dissociated, either by blocking NOS post-synaptically or knocking out GluA1 (Hardingham and Fox, 2006). When a NOS inhibitor is introduced to the post-synaptic neuron via the electrode, plasticity proceeds by changes in quantal amplitude without changes in the variance of the response amplitude (Hardingham and Fox, 2006). Similarly, where single or double quantal release peaks are isolated in layer 2/3 neuones, LTP occurs by changing the quantal amplitude without changes in release-probability (**Figure 6**). Conversely, in GluA1 knockouts, LTP results in changes in release-probability (NP_r) without changes in quantal amplitude (Q) (Hardingham and Fox, 2006). Given that potentiation is NO-dependent in GluA1 knockouts this implies that NO acts via a pre-synaptic mechanism in neocortex (Hardingham and Fox, 2006).

The situation is similar in the mature hippocampus, in that the plasticity present in the GluA1 knockouts is largely NO-dependent (Phillips et al., 2008; Romberg et al., 2009), but it is not clear in this case whether the locus of NO-dependent plasticity is pre- or post-synaptic, or perhaps both. Phillips et al. (2008) suggested a pre-synaptic origin for NO-dependent LTP based on the decrease in PPR for 14/21 cases following potentiation, while Romberg et al. (2009) found no change in average PPR. As noted above, it may be that the initial release-probability present at a particular connection affects the likelihood of pre-synaptic plasticity at that synapse (see section The Effect of Initial Release-Probability on the Locus of Plasticity).

Nitric oxide is also known to affect post-synaptic AMPA receptor trafficking; NO increases GluA1 insertion acting via sGC and protein kinase G (PKG) (Serulle et al., 2008) and GluA2 heteromer insertion by production of nitrosothiol groups on NSF (*N*-ethylmaleimide-sensitive factor) (Huang et al., 2005). Furthermore, endogenous NSF does appear to contain nitrosothiol groups *in vivo*. (Huang et al., 2005). However, the GluA1 insertion mechanism cannot be the one operating in the GluA1 knockouts, leaving the GluA2 mechanism as the most likely to be operating in these studies. This view is given further support by

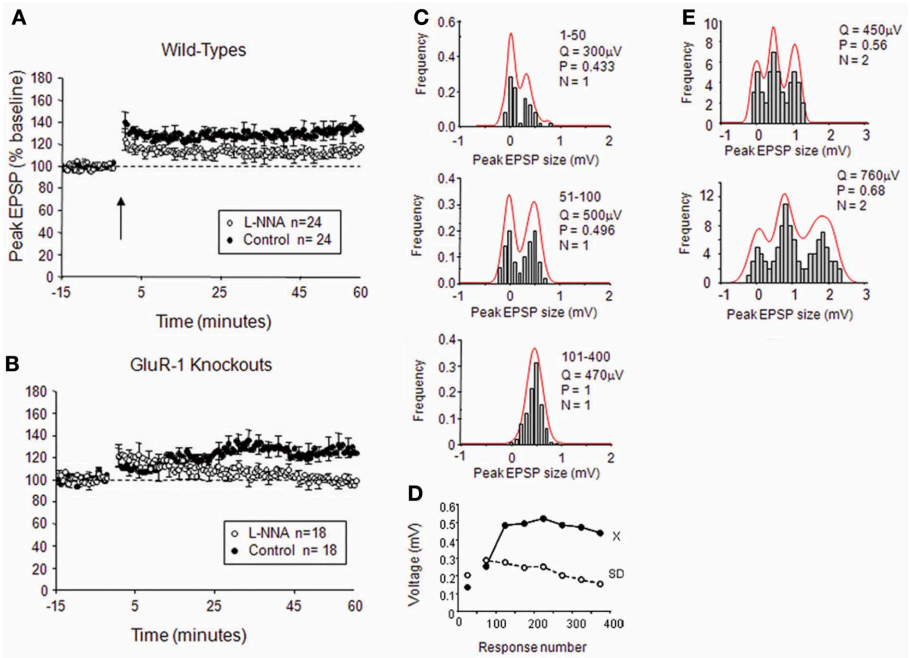

FIGURE 6 | Effects of NO on release-probability in cortical LTP. (A) Intracellular application of the NOS antagonist L-NNA reduces but does not abolish spike pairing LTP in wild-type mice. **(B)** Intracellular application of L-NNA abolishes LTP in mice lacking the GluA1 subunit of the AMPA receptor. **(C)** Examples of quantal analysis from a single release site input onto a layer II/III neuron from a wild-type mouse; note that LTP occurs by an increase in release-probability and quantal amplitude. **(D)** The plot of EPSP amplitude and standard deviation for the example in **(C)** during the course of LTP (x = mean, SD = standard deviation). **(E)** Example of quantal analysis from a double release site case in a wild-type treated with intracellular L-NNA; note that LTP occurs largely by an increase in quantal amplitude with a minor increase in release-probability. Q is quantal amplitude, P is release-probability, and N is the number of release sites. Adapted from Hardingham and Fox (2006) with permission of the Society for Neuroscience.

data showing the PKC dependence of LTP in the GluA1 knockout animals (Romberg et al., 2009). Nevertheless, in wild-types it is possible that both GluA1 and GluA2 are controlled by NO signaling. Accumulation of cGMP in hippocampal cells has recently been demonstrated using NO donors (Bartus et al., 2013) giving further credence to a post-synaptic role for NO. Furthermore, there is some evidence that dexras1 is activated by NO and is located post-synaptically due to CAPON binding dexras1 and NOS1 (Fang et al., 2000; Cheah et al., 2006). Together with the substantial evidence that NO acts pre-synaptically (Section NO Controls Pre-Synaptic Function), these findings raise the intriguing possibility that NO might play a role in coordinating pre- and post-synaptic changes at excitatory synapses during plasticity.

EVIDENCE FOR THE ROLE OF NO IN EXPERIENCE-DEPENDENT PLASTICITY

There is an extensive literature on the role of NO in learning and memory. Peripheral administration of NOS inhibitors have been shown to impair spatial memory acquisition or recall (Bohme et al., 1993; Chapman et al., 1992; Zou et al., 1998; Majlessi et al., 2008), social interactions (Bohme et al., 1993) and object recognition memory (Cobb et al., 1995). Central administration of NOS antagonists also alters behavior, including spatial learning in the Morris water maze and the passive avoidance test (Qiang et al., 1997; Majlessi et al., 2008; Li et al., 2012), arguing against the peripheral effects of the drug. Inhibitors more specific to NOS1 have also shown sensitivity to behavioral performance in spatial reference and working memory (Holscher et al., 1996; Zou et al., 1998; Yildiz Akar et al., 2009). Furthermore, NOS1 knockout mice show impaired spatial memory, social interactions and contextual fear memory (Weitzdoerfer et al., 2004; Kelley et al., 2009; Tanda et al., 2009). In contrast, NOS3 knockout mice exhibit enhanced spatial learning, retention and reversal learning in the Morris water maze but increased anxiety-like behaviors in the plus maze and the open arena (Frisch et al., 2000). However, spatial learning is comparable to controls in the radial arm maze (Dere et al., 2001), suggesting that NOS3 knockout confers a specific deficit in spatial learning and may therefore play a particular role in hippocampal plasticity, where it has been shown to play a role in LTP in concert with NOS1 (Hopper and Garthwaite, 2006; Phillips et al., 2008).

A simpler form of experience-dependent plasticity that can be quantified by measuring neuronal responses rather than behavior is the plasticity that results from whisker deprivation in the barrel cortex. Depriving a single whisker for several days leads to expansion of the area of cortex dominated by that whisker (Fox, 1992; Wallace and Fox, 1999). NO is implicated in the potentiation component of this plasticity as αNOS1 knockouts exhibited reduced single whisker potentiation (Dachtler et al., 2011). In parallel with the LTP studies (Hardingham and Fox,

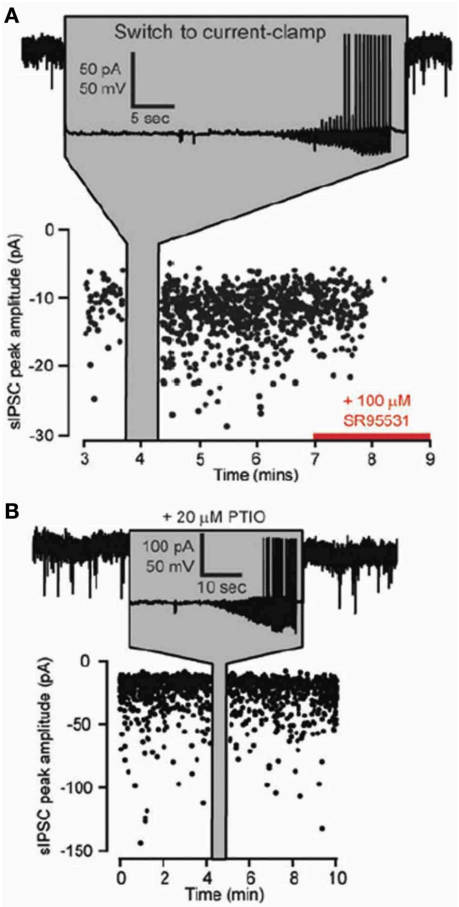

FIGURE 7 | Effects of NO on the frequency of GABAergic spontaneous IPSCs (sIPSCs). (A) Plot of the peak amplitudes of spontaneous IPSCs against time for a thalamocortical (TC) lateral geniculate neuron. At the time indicated by the gray panel a switch is made from voltage to current clamp so that somatic action potentials can be generated. Note the increase in sIPSCs on returning to voltage clamp and the block of all sIPSCs by SR95531 toward the end of the experiment. **(B)** A similar recording from a TC relay neuron with the same protocol as in **(A)**, but in the presence of the NO scavenger PTIO (20 μM) in the external solution. Note that GABAergic synaptic plasticity is blocked. Adapted from Bright and Brickley (2008) with kind permission of the authors and the Physiological Society.

2006), experience-dependent potentiation was only abolished in double knockouts of αNOS1 and GluA1 (Dachtler et al., 2011). Plasticity was present in double knockouts of NOS3 and GluA1, suggesting that αNOS1 is the important isoform in the cortex, probably due to the close association between αNOS1 and the NMDA receptor (see section The Cellular Location of NOS1). In further support of this idea, NMDA-dependent release of NO is impaired in αNOS1 but not NOS3 knockout mice (Dachtler et al., 2011).

Further analysis of plasticity in αNOS1 knockout mice reveals both LTP and experience-dependent potentiation are abolished in male but not female mice (Dachtler et al., 2012). This could either mean that male mice rely solely on NO-dependent forms of potentiation, or that some form of compensation for the lack of NOS1 takes place in the female knockout mice that does not occur in the males. The sex difference was not seen in wild-type animals suggesting that the latter is a possible explanation. The sex difference in the αNOS1 knockout mice may be of importance to interpreting stroke data because factors involved in LTP are often also involved in excitotoxicity. NOS1 has long been known to be a factor in ischemic damage in stroke (Huang et al., 1994), most likely through the association of αNOS1 and PSD-95 (Cao et al., 2005). However, the magnitude of ischemic damage differs depending upon sex. Male αNOS1 knockout mice have less ischemic damage than wild-types, while female αNOS1 knockout mice have more damage than their wild-type counterparts (McCullough et al., 2005).

NO AND PLASTICITY AT GABAERGIC SYNAPSES

Because NO can play a role in pre-synaptic plasticity, it also means that it is not restricted to act on a particular set of post-synaptic receptors or the protein trafficking machinery associated with them. Instead, in so far as the vesicular release machinery is common across transmitter systems, NO can potentially regulate release for several different neurotransmitters including GABA (**Table 2**).

Anatomical evidence implicates NO in regulation of pre-synaptic GABA release. In excitatory pyramidal cells in the hippocampus, NOS1 lies post synaptic to GABAergic synapses and the "NO receptor" (sGC) lies in the pre-synaptic terminals of those same GABAergic synapses, thereby providing both elements required for retrograde synaptic signaling in close assembly (Szabadits et al., 2007). In this case, rather than being associated with PSD95, which does not appear to localize at post-synaptic densities of symmetric synapses, GRIP1 may bind NOS1 at the post-synaptic site. The pre-synaptic terminals in question belong to parvalbumin- and CCK-containing cells that synapse onto somata and proximal dendrites of pyramidal cells. Consistent with this location, application of NO donors increases cGMP levels in GABAergic interneurons (Bartus et al., 2013). It is not clear how the endogenous signal arises to activate NOS1 at these inhibitory synapses, but one possibility is that action potentials could raise intracellular calcium via voltage gated calcium channels and the spatial localization of NOS1 immediately post-synaptic to the GABAergic terminals targets NO to the inhibitory terminals.

Physiological evidence further implicates NO in GABAergic synaptic plasticity. Evidence comes from observations on paraventricular neurons (Li et al., 2002), the prepositus hypoglossal nucleus (Moreno-Lopez et al., 2002), the amygdala (Lange et al., 2012) and thalamic projection neurons (Bright and Brickley, 2008). In the hippocampus, NMDA receptor activation in pyramidal cells causes an increase in spontaneous $GABA_A$ receptor mediated IPSCs that are sensitive to an NO scavenger (Xue et al., 2011). In the ventral tegmental area, GABAergic synapses onto dopaminergic neurons express a pre-synaptic form of LTP that is dependent upon NMDA receptor activation, NO, GC, and PKG for its induction and maintenance and is selective to $GABA_A$ synapses (Nugent et al., 2007, 2009). Furthermore, pre-synaptic GABAergic LTP from the lateral amygdala to the basolateral amygdala depends upon NO generated from glutamatergic neurons (Lange et al., 2012).

Table 2 | The role of Nitric oxide in GABAergic function.

References	Title	Presynaptic action?	Retrograde messenger?	Effect?	Structure (preparation)
Lange et al., 2012	Heterosynaptic long-term potentiation at interneuron-principal neuron synapses in the amygdala requires nitric oxide signaling	✓	✓	Effect on plasticity	Amygdala (slice)
Moreno-Lopez et al., 2002	Nitric oxide facilitates GABAergic neurotransmission in the cat oculomotor system: a physiological mechanism in eye movement control	✓	✓	Controls velocity responsiveness of PH neurons	Medial vestibular nucleus projection to prepositus hyperglossi (PH) neurons (*in vivo*)
Szabadits et al., 2007	Hippocampal GABAergic synapses possess the molecular machinery for retrograde nitric oxide signaling	✓	✓	Anatomical evidence: nNOS is post and sCG presynaptic	Hippocampus (*in vivo*)
Xue et al., 2011	NMDA receptor activation enhances inhibitory GABAergic transmission onto hippocampal pyramidal neurons via presynaptic and postsynaptic mechanisms	✓	✓	Increase in sIPSP frequency and amplitude	Hippocampal (slice)
Yang et al., 2007	Kv1.1/1.2 channels are downstream effectors of nitric oxide on synaptic GABA release to preautonomic neurons in the paraventricular nucleus	✓	✓	Nitric oxide acts on GABA via Kv1.1/1.2	Paraventricular nucleus of the hypothalamus (slices)
Yang et al., 2007	Kv1.1/1.2 channels are downstream effectors of nitric oxide on synaptic GABA release to preautonomic neurons in the paraventricular nucleus	✓	✓	Increases frequency but not amplitude of inhibitory minis	Paraventricular nucleus of the hypothalamus (slices)
Bright and Brickley, 2008	Acting locally but sensing globally: impact of GABAergic synaptic plasticity on phasic and tonic inhibition in the thalamus	✓	✓	Increases frequency of sIPSCs	Thalamus (slices)
Wall, 2003	Endogenous nitric oxide modulates GABAergic transmission to granule cells in adult rat cerebellum	✓	✓	NO modulates toninc GABA release	Cerebellum (slices)
Holmgren and Zilberter, 2001	Coincident spiking activity induces long-term changes in inhibition of neocortical pyramidal cells			Analogous to cases where nitric oxide is involved	Neocortical (slices)

The studies on GABAergic potentiation in the thalamus are particularly interesting because it only requires post-synaptic action potentials, which cause an increase in spontaneous GABAergic mIPSC frequency. This effect is blocked by the NO scavenger PTIO (Bright and Brickley, 2008) suggesting that the action potentials lead to release of NO that in turn produces changes in GABA release (**Figure 7**). The NO donor SNAP can also increase GABA mini frequency in these cells (Bright and Brickley, 2008). The sufficiency of post-synaptic action potentials in this study is reminiscent of the findings of Volgushev and colleagues in the visual cortex, who showed that post-synaptic action potentials produced NO-dependent potentiation in pyramidal cells (Volgushev et al., 2000) and Phillips et al. who showed that NO-dependent LTP in the hippocampus relies on somatic post-synaptic action potentials (Phillips et al., 2008). These findings raise the possibility that post-synaptic action potentials may simultaneously produce NO-dependent plasticity at inhibitory synapses and spike timing-dependent plasticity at excitatory synapses on the same cell, a property that may be involved in maintenance of inhibitory-excitatory balance.

NO AND THE REGULATION OF EXCITATORY/INHIBITORY BALANCE

Cells in the cortex exhibit a balance between excitation and inhibition such the ratio between inhibitory and excitatory conductances is relatively constant for different inputs. For layer 5 pyramidal cells in the visual cortex, the ratio of excitatory to inhibitory conductance has been estimated at 20:80 (Le Roux et al., 2006) using the method of Monier et al. (2008). The

excitatory/inhibitory (E/I) balance would be expected to be a universal phenomenon as loss of E/I balance in favor of excitation leads to epilepsy. Consistent with this idea, inhibition has been shown to be matched to excitation in the visual (Anderson et al., 2000; Priebe and Ferster, 2005) auditory (Wehr and Zador, 2003, 2005) and somatosensory cortices (Wilent and Contreras, 2005). Studies have shown that NO may play a role in the maintenance of the E/I balance in the visual cortex. It can be demonstrated that the E/I balance is maintained in layer 5 pyramidal cells following potentiation by theta burst stimulation of cortical layers 2/3, 4 or 6 (Le Roux et al., 2006). Stimulating layer 4 and increasing endogenous levels of NO by dosing a cortical slice with L-arginine, or administration of an NO donor, also increases excitatory and inhibitory conductances in balance (Le Roux et al., 2009). These studies suggests that NO may play a homeostatic role in maintaining the balance between excitation and inhibition in the cortex.

CONCLUSIONS

Deciphering the role of NO in the brain has not been a simple matter and at times the results of different studies have been confusing. Nevertheless, a clearer picture is now emerging of how NO might act to regulate synaptic function in the brain. In excitatory cells, NOS1 is located discretely in spines and is tethered to the post-synaptic membrane by its interaction with PSD95 in complete contrast to its location in a subpopulation of $NOS1^+$ NPY^+ inhibitory cells, where NOS1 is located in the cytoplasm along axons and dendrites and appears to be expressed at higher levels. The low levels of NOS1 expression in excitatory cells of the cortex and hippocampus dictate that under normal physiological conditions low concentrations of NO are evolved during stimulation by calcium, which in turn means that it has a relatively small range and is therefore probably synapse-specific in its action. The only obvious receptor that is sensitive at the low nM to pM range is guanylate cyclase, although there is some evidence for endogenous levels of proteins with nitrosothiol groups that would require higher concentrations of NO. There is a substantial body of literature that suggests that NO acts in a retrograde manner on several aspects of vesicular release and recycling, so much so that it would seem perverse to argue that NO does not act pre-synaptically at this point. Present evidence suggests that NO acts in a retrograde manner to affect not only glutamatergic synapses but also GABAergic synapses as well as other transmitter systems. Finally, there is substantial evidence in the literature that the retrograde route of action is important for plasticity in the cortex and hippocampus in both inhibitory and excitatory cells.

Nevertheless, a number of important questions remain about the action of NO at synapses. Two questions relate to the concentration of NO in the brain. First, are the levels of NO required for nitrosothiol production at the SNARE complex proteins actually achieved *in vivo*? Second, could higher levels of NO reported in some studies be generated by the higher NOS levels present in the $NOS1^+$ GABAergic cells? A further set of questions relate to the action of NO at pre-synaptic GABAergic synapses. There is evidence that sGC is present in GABAergic terminals and that NOS1 lies post-synaptic to it (Szabadits et al., 2007). There is evidence that GABAergic mini EPSC frequency increases following somatic spiking in the LGn (Bright and Brickley, 2008). Therefore, what is the mechanism of post-synaptic spike-dependent potentiation of GABAergic transmission and is it indeed NO-dependent in the cortex and hippocampus? More generally, is this mechanism related to the post-synaptic spike potentiation present at excitatory synapses (Volgushev et al., 2000)? Unraveling this effect could help us understand whether the E/I balance is maintained by NO acting simultaneously on GABAergic and excitatory transmission (Le Roux et al., 2009). Finally, while we have concentrated on the pre-synaptic role of NO in this review, there is evidence that NO also has a post-synaptic action. In addition to activation of post-synaptic sGC, there is evidence that post-synaptic proteins have nitrosothiol groups, particularly those close to its PSD location (see **Figure 1**). If NO also has a post-synaptic role in plasticity it raises the additional question about whether it can play a homeostatic role in balancing or matching pre- and post-synaptic function. With a little good fortune, it will not take another 25 years of research to solve these and other related questions on the role NO plays in synaptic function in the brain.

ACKNOWLEDGMENTS

We should like to acknowledge the support of the MRC to Kevin Fox for the authors' own work. We should also like to thank John Garthwaite for critically reading an earlier draft of the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 13 August 2013; paper pending published: 11 September 2013; accepted: 05 October 2013; published online: 31 October 2013.
Citation: Hardingham N, Dachtler J and Fox K (2013) The role of nitric oxide in pre-synaptic plasticity and homeostasis. Front. Cell. Neurosci. 7:190. doi: 10.3389/fncel.2013.00190
This article was submitted to the journal Frontiers in Cellular Neuroscience.

frontiers in
CELLULAR NEUROSCIENCE

REVIEW ARTICLE
published: 14 February 2014
doi: 10.3389/fncel.2014.00040

How voltage-gated calcium channels gate forms of homeostatic synaptic plasticity

C. Andrew Frank*

Department of Anatomy and Cell Biology, University of Iowa Carver College of Medicine, Iowa City, IA, USA

Edited by:
Jaichandar Subramanian, Massachusetts Institute of Technology, USA

Reviewed by:
Rheinallt Parri, Aston University, UK
Ping Liu, University of Connecticut Health Center, USA

***Correspondence:**
C. Andrew Frank, Department of Anatomy and Cell Biology, University of Iowa Carver College of Medicine, 51 Newton Road, 1-661A Bowen Science Building, Iowa City, IA 52242, USA
e-mail: andy-frank@uiowa.edu

Throughout life, animals face a variety of challenges such as developmental growth, the presence of toxins, or changes in temperature. Neuronal circuits and synapses respond to challenges by executing an array of neuroplasticity paradigms. Some paradigms allow neurons to up- or downregulate activity outputs, while countervailing ones ensure that outputs remain within appropriate physiological ranges. A growing body of evidence suggests that homeostatic synaptic plasticity (HSP) is critical in the latter case. Voltage-gated calcium channels gate forms of HSP. Presynaptically, the aggregate data show that when synapse activity is weakened, homeostatic signaling systems can act to correct impairments, in part by increasing calcium influx through presynaptic Ca_V2-type channels. Increased calcium influx is often accompanied by parallel increases in the size of active zones and the size of the readily releasable pool of presynaptic vesicles. These changes coincide with homeostatic enhancements of neurotransmitter release. Postsynaptically, there is a great deal of evidence that reduced network activity and loss of calcium influx through Ca_V1-type calcium channels also results in adaptive homeostatic signaling. Some adaptations drive presynaptic enhancements of vesicle pool size and turnover rate via retrograde signaling, as well as *de novo* insertion of postsynaptic neurotransmitter receptors. Enhanced calcium influx through Ca_V1 after network activation or single cell stimulation can elicit the opposite response—homeostatic depression via removal of excitatory receptors. There exist intriguing links between HSP and calcium channelopathies—such as forms of epilepsy, migraine, ataxia, and myasthenia. The episodic nature of some of these disorders suggests alternating periods of stable and unstable function. Uncovering information about how calcium channels are regulated in the context of HSP could be relevant toward understanding these and other disorders.

Keywords: homeostatic synaptic plasticity, VGCCs, Ca_V1 channels, Ca_V2 channels, neurotransmitter release, synaptic scaling, synaptic growth, calcium channelopathies

INTRODUCTION

Many forms of neuroplasticity drive changes in synaptic outputs, and they are thought to underlie fundamental neurological phenomena, like learning. At the same time, stabilizing forms of neuroplasticity—collectively termed homeostatic synaptic plasticity (HSP)—work to ensure that neuronal outputs are maintained within physiologically appropriate levels. The study of HSP has shed considerable light on how neuronal stability is maintained. Perturbations of synaptic function can trigger homeostatic modulations in activity parameters such as presynaptic neurotransmitter release, neurotransmitter receptor expression, ion channel density, or conductance properties (Pérez-Otaño and Ehlers, 2005; Davis, 2006, 2013; Marder and Goaillard, 2006; Turrigiano, 2008; Maffei and Fontanini, 2009; Pozo and Goda, 2010; Watt and Desai, 2010; Marder, 2012; Turrigiano, 2012). The underlying molecular mechanisms that enable such change are critical (Lazarevic et al., 2013). How exactly do synapses detect challenges to their activity and then engage biological homeostats to correct errors? Some progress has been made in answering this question using a variety of models such as *Drosophila melanogaster* (Davis, 2013; Frank, 2014), crustaceans (Marder and Goaillard, 2006; Marder and Bucher, 2007; Marder, 2012), and rodents (Chen et al., 2014; Lee et al., 2014; Pribiag and Stellwagen, 2014; Thalhammer and Cingolani, 2014; Wenner, 2014; Whitt et al., 2014). In recent years, voltage-gated calcium channels (VGCCs or Ca_V channels) have emerged as critical for homeostatic control of synapse function in several experimental contexts.

When considering how Ca_V channels control any process (such as HSP), it is important to consider auxiliary interacting proteins, cellular context, and the high degree to which Ca_V-driven processes are conserved across metazoans. It is known that neuronal Ca_V channels mediate cellular calcium entry and regulate activity-dependent processes such as neurotransmission, gene transcription, and intracellular signaling cascades (Catterall, 2000; Zamponi, 2005; Tedford and Zamponi, 2006; Catterall and Few, 2008; Dolphin, 2009; Currie, 2010; Lipscombe et al., 2013). Control of Ca_V function occurs at many levels, starting

with the regulation of channel subunit mRNA splicing and channel subunit trafficking (Lipscombe et al., 2013). Much has also been learned about the targeting and cellular distribution of Ca_V channels (Herlitze et al., 2003), as well as G-Protein-mediated inhibition of presynaptic Ca_V channels (Tedford and Zamponi, 2006; Currie, 2010; Zamponi and Currie, 2013). Across metazoans as diverse as nematodes and humans, cytoplasmic, calcium-binding regulatory messengers such as calmodulin integrate cytoplasmic calcium entry with activation of downstream targets, such as calcium/calmodulin-dependent protein kinases (e.g., CaMK, CaMKII) (Liu et al., 2007)—or even inhibition or facilitation of Ca_V channels themselves (Halling et al., 2006; Dunlap, 2007; Catterall and Few, 2008; Minor and Findeisen, 2010; Christel and Lee, 2012). Mitochondria and the endoplasmic reticulum also play conserved modulatory roles at many model synapses, acting as calcium buffers and intracellular sources of calcium (Verkhratsky and Petersen, 1998; Collin et al., 2005; Liu et al., 2005; Williams et al., 2013). Additionally, genetic mutations and toxins that impair Ca_V channel function cause numerous cellular defects, including impairment of neurotransmission and alterations of forms of neuroplasticity (Catterall and Few, 2008; Norton and McDonough, 2008). These topics have been studied and reviewed thoroughly elsewhere (including a comprehensive book, Zamponi, 2005).

With the backdrop of this considerable knowledge, we consider the emerging roles that VGCCs play in HSP. We review electrophysiological, biochemical, and imaging data that have established the important roles Ca_V channels play in multiple forms of HSP across diverse experimental systems (**Figures 1, 2**). We also briefly consider the idea that Ca_V channels might link HSP to disorders in which underlying neuronal stability is lost.

CA_V2 CHANNELS AND PRESYNAPTIC HOMEOSTATIC SYNAPTIC PLASTICITY

Ca_V2-type calcium channels function in the presynaptic nervous systems of nearly all animals. There are several Ca_V2 subtypes, classified primarily by sensitivity to different toxins; they include the P/Q- ($Ca_V2.1$), N- ($Ca_V2.2$), and R-type ($Ca_V2.3$) calcium channels (Catterall et al., 2005; Zamponi, 2005). Ca_V2 channels generally mediate fast neurotransmission, gating presynaptic calcium influx upon cellular depolarization (Catterall et al., 2005; Zamponi, 2005). In turn, calcium-sensing molecules such as Synaptotagmin I trigger rapid evoked neurotransmitter release (Giraudo et al., 2006; Schaub et al., 2006; Tang et al., 2006). Here we review data that demonstrate roles for presynaptic Ca_V2 channels in HSP at fruit fly and mammalian synapses. In particular, several forms of homeostatic plasticity appear to govern alterations in presynaptic neurotransmitter release by directly targeting the amount of terminal calcium influx through Ca_V2-type channels.

HOMEOSTATIC PLASTICITY AND CA_V2 AT THE DROSOPHILA NEUROMUSCULAR SYNAPSE

The *Drosophila melanogaster* larval neuromuscular junction (NMJ) is a glutamatergic synapse that exhibits a strong capacity

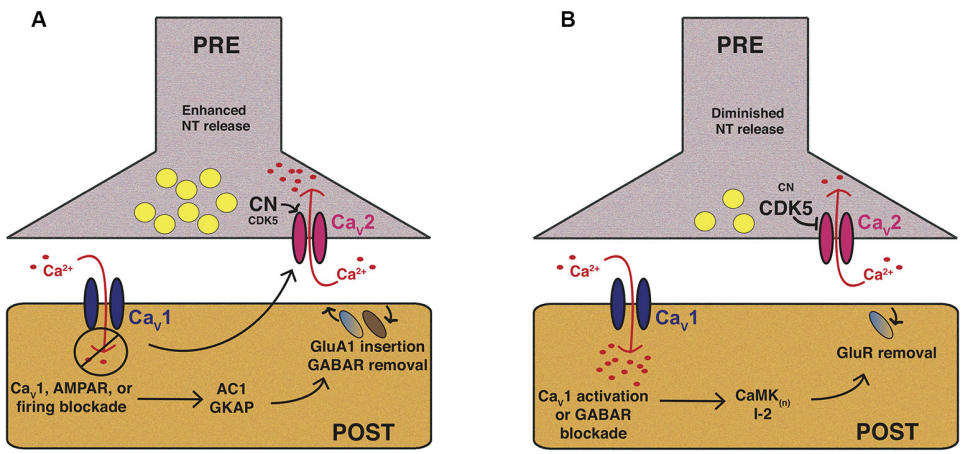

FIGURE 1 | Mammalian Ca_V1 and Ca_V2 channels play central roles in forms of homeostatic plasticity. These two cartoons attempt to synthesize knowledge of mammalian pre- and postsynaptic homeostatic plasticity mechanisms involving Ca_V1 and Ca_V2 calcium channels in preparations like cultured hippocampal neurons. The cartoons are not intended to depict a single synaptic preparation or universally conserved mechanism, though some molecular responses may be widely conserved. **(A)** Homeostatic potentiation of synapse function. Inhibition of synaptic activity or postsynaptic Ca_V1 calcium influx results in multiple changes, including postsynaptic signaling through molecules like adenylate cyclase 1 (AC1) or guanylate kinase-associated protein (GKAP) to drive activating mechanisms, such as glutamate receptor insertion. Trans-synaptic signaling controlled by factors like Target of Rapamycin (TOR) and Brain-Derived Neurotrophic Factor (BDNF) can trigger enhanced presynaptic release probability. From a variety of systems there is evidence for enhanced presynaptic calcium influx through Ca_V2—which may require diminishment of cyclin-dependent kinase 5 (CDK5) function—as well as an enhanced readily releasable pool of presynaptic vesicles. **(B)** Homeostatic downscaling of synapse function. Synaptic activation (e.g., through GABA receptor blockade) and/or enhanced postsynaptic calcium influx through Ca_V1 results in the activation of diverse pathways, such as those mediated by calcium/calmodulin-dependent kinases (CaMK), as well as the Protein Phosphatase 1 (PP1) inhibitor, I-2. This can result in removal of excitatory glutamate receptors from the synapse. Presynaptically, there is evidence of diminished calcium influx through Ca_V2, and thus, diminished evoked presynaptic release.

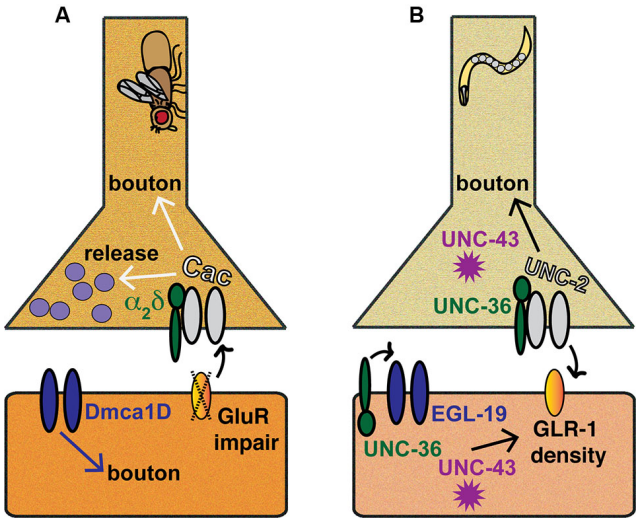

FIGURE 2 | Invertebrate models of Ca_V-directed homeostatic plasticity. Inspection of the *Drosophila melanogaster* NMJ has provided a wealth of information regarding the roles VGCCs and associated molecules play in homeostatic plasticity, as has examination of *C. elegans* preparations, such as the ventral nerve cord (VNC) or the NMJ. **(A)** Drosophila NMJ. Pharmacological or genetic impairment of postsynaptic glutamate receptors triggers a retrograde signaling process that results in enhanced presynaptic Cac/Ca_V2 function and increased neurotransmitter release. Additionally, Cac/Ca_V2, $\alpha_2\delta$, and Dmca1D/Ca_V1 all affect synaptic bouton development or maturation at the NMJ. **(B)** *C. elegans* synapses. At the *C. elegans* VNC, the coordinated functions of UNC-2/Ca_V2, EGL-19/Ca_V1, UNC-36/$\alpha_2\delta$ and UNC-43/CaMKII ensure proper coupling of GLR-1 glutamate receptor density to developmental growth.

for homeostatic regulation (Frank, 2014; **Figure 2A**). Several studies have established that the NMJ retains normal levels of postsynaptic evoked excitation, even when it is challenged by chronic or acute perturbations to excitability (Petersen et al., 1997; Davis et al., 1998; DiAntonio et al., 1999; Paradis et al., 2001; Frank et al., 2006). For instance, genetic or pharmacological impairment of muscle glutamate receptors results in decreased sensitivity to single vesicles of glutamate (Petersen et al., 1997; Frank et al., 2006). A homeostatic, retrograde, muscle-to-nerve signaling process helps to offset this decreased sensitivity by inducing a presynaptic increase in vesicle release. This increase has been measured by multiple electrophysiological means (Petersen et al., 1997; Frank et al., 2006). We recently reviewed our understanding of mechanisms underlying homeostatic plasticity at the Drosophila NMJ, including investigations to uncover the unknown retrograde signal(s) and the central roles played by Ca_V2 channels and presynaptic calcium influx (Frank, 2014). For completeness, some of that same information is reiterated here, but it is updated with newly published data.

Cacophony/Ca_V2

The pore-forming α_1 subunit of Drosophila Ca_V2 channels is called Cacophony (Cac; Smith et al., 1996). Loss-of-function mutations in the *cac* gene were originally found in the 1970s from genetic approaches to identify fruit flies with visual defects and defects in mating behavior; indeed, partial loss-of-function *cac* mutant males buzz their wings with a defective, "cacophonous" mating song (Kulkarni and Hall, 1987; Smith et al., 1998). Cac is required throughout much of development. Null *cac* mutant embryos fail to hatch (Kulkarni and Hall, 1987; Kurshan et al., 2009), and *cac* mRNA expression is prominent in the nervous system (Smith et al., 1996). Partial loss-of-function *cac* hypomorphs are viable and fertile, permitting synaptic analyses at larval and adult stages (Smith et al., 1998; Kawasaki et al., 2000; Brooks et al., 2003). Hypomorphic *cac* mutations cause pronounced neurotransmission defects (Kawasaki et al., 2000; Brooks et al., 2003; Frank et al., 2006), and at the third instar larval stage of development, some hypomorphs display mild NMJ growth defects (Rieckhof et al., 2003; Xing et al., 2005). Neuronally expressed Cac-Green Fluorescent Protein (GFP) rescues the lethality of nulls and co-localizes with presynaptic active zone markers (Kawasaki et al., 2004). Transgenic Cac-GFP has proven to be a useful tool in examining active zones and associated proteins (as in Fouquet et al., 2009).

Ca_V2-type calcium channels also play a critical role in Drosophila NMJ homeostasis. When the NMJs of *cac* hypomorphs are challenged with a pharmacological or genetic impairment of postsynaptic glutamate receptor function, there is no increase in presynaptic neurotransmitter release (Frank et al., 2006). Therefore, not only do *cac* mutants have neurotransmission defects, but the NMJ also fails to maintain muscle excitation at baseline levels (i.e., the already impaired *cac* mutant levels). This phenotype constitutes a block of synaptic homeostasis. One concern about this finding is that *any* mutant with baseline neurotransmission defects might also have defects in the ability to respond to homeostatic challenges. However, it has been demonstrated that several Drosophila mutations cause baseline NMJ neurotransmission defects without impairing homeostatic plasticity (Goold and Davis, 2007; Dickman and Davis, 2009; Younger et al., 2013). Conversely, other mutations or gene knockdowns impair homeostatic plasticity without causing baseline neurotransmission defects (Dickman and Davis, 2009; Marie et al., 2010; Müller et al., 2011; Dickman et al., 2012).

The requirements for Cac/Ca_V2 during NMJ homeostasis correspond to measureable changes in presynaptic calcium influx. Direct measurement of evoked presynaptic calcium transients reveals that chronic and acute glutamate receptor impairments induce significant (23–30%) increases in presynaptic calcium influx, presumably through Cac/Ca_V2 channels (Müller and Davis, 2012). A hypomorphic *cac* point mutation, cac^S, blocks this increase (Müller and Davis, 2012). In addition, pharmacological inhibition of the glutamate receptors results in a swift increase in expression of the presynaptic active zone protein Bruchpilot (an ELKS/CAST homolog), as well as an increase in the active zone cytomatrix structure (Weyhersmüller et al., 2011). Together, these results suggest that Ca_V2 and other active zone proteins are important targets for homeostatic signaling processes. This is logical because small changes in presynaptic calcium influx are well known to correspond to large changes in vesicle release and neurotransmission (Katz and Miledi, 1970).

What might enhance Ca_V2 function during HSP at the NMJ? Genetic, electrophysiological, and calcium imaging data suggest

that presynaptic Ca_V2 may be targeted by several signaling paradigms that reside on both sides of the synapse. This is not surprising, given that retrograde, muscle-to-nerve signaling governs synaptic homeostasis at the NMJ. What is surprising is how many pieces of this signaling puzzle have emerged in a few short years. Linking those pieces together into a logical framework will be critical as research moves forward.

Postsynaptic signaling and Ca_V2

Following genetic glutamate receptor impairment, a protein-translation-dependent signaling process in the muscle is driven by Drosophila Target of Rapamycin (TOR) and S6 kinase (S6K) to potentiate presynaptic release (Penney et al., 2012). By contrast, two negative postsynaptic signaling factors are the nuclear receptor Importin 13 (Imp13) and the muscle cytoskeletal/matrix support molecule Dystrophin. Impairments of Imp13 or Dystrophin function cause an increase in presynaptic vesicle release (Giagtzoglou et al., 2009; Pilgram et al., 2010, 2011). Further, postsynaptic *imp13* loss of function causes an increase in presynaptic intracellular calcium levels (Giagtzoglou et al., 2009). It is not known if S6K/TOR-, Dystrophin-, and Imp13-mediated signaling events directly interact with one another to regulate homeostatic changes in neurotransmitter release. Given the extensive Drosophila genetic toolkit, this issue is imminently addressable.

Presynaptic signaling and Ca_V2

On the presynaptic side of the synapse, several molecules are hypothesized to directly or indirectly enhance Ca_V2 function after glutamate receptor impairment. Among these is a signaling system driven by the Drosophila Eph receptor tyrosine kinase, the cytoplasmic guanine exchange factor Ephexin, and Rho-type GTPases (Frank et al., 2009). Loss-of-function mutations in components of this signaling system impair synaptic homeostasis and interact genetically with *cac* mutations (Frank et al., 2009).

The kinesin super family member Khc-73 may work in a similar fashion as Eph/Ephexin. *khc-73* loss-of-function mutations completely impair synaptic homeostasis (Tsurudome et al., 2010), and electron microscopy data demonstrate that Khc-73 contributes to enhancement of active zone components at the NMJ (Tsurudome et al., 2010). A negative regulator of *khc-73* gene expression is the miR-310 micro RNA cluster. Loss of the miR-310 genetic locus phenocopies transgenic overexpression of *khc-73* and shows enhanced presynaptic evoked calcium transients, possibly due to enhanced Ca_V2 activity (Tsurudome et al., 2010).

Finally, a factor that interacts with calcium channels and assists their localization to the active zone is Drosophila Rab3 Interacting Molecule (RIM; Graf et al., 2012). *rim* loss-of-function mutations block synaptic homeostasis at the NMJ, but not because of a failure to upregulate presynaptic calcium influx. Instead, *rim* mutations occlude important increases in the size of the readily releasable pool (RRP) of presynaptic vesicles (Müller et al., 2012).

New data: ENaC and Ca_V2

A very recent study offers compelling insights into how Ca_V2 function may be potentiated during synaptic homeostasis. This study demonstrates that presynaptic Epithelial Sodium Channel (ENaC) components encoded by the *pickpocket11* (*ppk11*) and *pickpocket16* (*ppk16*) genes are both required for synaptic homeostasis (Younger et al., 2013). They are also required for the accompanying enhanced presynaptic calcium influx (Younger et al., 2013). These results are drawn not only from genetic mutant data, but also from application of the drug benzamil to impair ENaC (Younger et al., 2013). Based on these new data and previously published data about ENaC function as a voltage-insensitive cation channel (Schild, 2010), one compelling model is that PPK11- and PPK16-ENaC channels act to depolarize presynaptic membrane voltage (Younger et al., 2013). As a result, depolarized resting presynaptic voltage may enhance presynaptic Ca_V2 activity during homeostatic plasticity.

Arclight is a new genetically encoded voltage sensor that shows robust utility in Drosophila to measure membrane voltage in response to action potentials and sub threshold events (Cao et al., 2013). An attractive possibility for future research is to design new tools—similar to Arclight—that could reliably measure alterations in resting membrane voltage. Such tools could be utilized to directly test the voltage/Ca_V2 hypothesis for ENaC and possibly to hunt for other factors that control neurotransmission and HSP upstream of Ca_V2 function.

CA_V2 AND HOMEOSTATIC SYNAPTIC PLASTICITY AT MAMMALIAN CENTRAL SYNAPSES

There are similarities in Ca_V2-gated HSP between the Drosophila NMJ (**Figure 2A**) and mammalian central synapses (**Figure 1**). Cultured rodent neurons possess a strong ability to maintain a set point of activity through a homeostatic process termed synaptic scaling (Turrigiano, 2012; Chen et al., 2014; Lee et al., 2014; Pribiag and Stellwagen, 2014; Siddoway et al., 2014; Thalhammer and Cingolani, 2014). It is known that the drugs tetrodotoxin (TTX), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), and 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX) abolish neuronal firing and transmission. TTX blocks sodium channels (Narahashi, 2008), while CNQX and NBQX block postsynaptic AMPA glutamate receptors (Honore et al., 1988; Sheardown, 1993). Conversely, drugs like bicuculline or gabazine inhibit γ-aminobutyric acid (GABA) receptor-mediated inhibitory neurotransmission, and thus, increase activity (Curtis et al., 1970; Uchida et al., 1996). A landmark finding was that chronic application (>48 h) of TTX, CNQX, or bicuculline to cultured rodent visual cortical neurons or to spinal cord neurons elicits a scaling up (TTX and CNQX) or scaling down (bicuculline) of spontaneous miniature amplitudes (O'Brien et al., 1998; Turrigiano et al., 1998). Additionally, drug applications change firing rates in the short run, but upon TTX/CNQX washout or prolonged exposure to bicuculline, activity parameters in exposed neurons homeostatically drive firing rates back in the opposite direction (Turrigiano et al., 1998).

Much work has illuminated the postsynaptic events that accompany scaling—namely, altered postsynaptic neurotransmitter receptor composition and sensitivity to glutamate (O'Brien et al., 1998; Turrigiano et al., 1998; Wierenga et al., 2005; Sutton et al., 2006; Turrigiano, 2012). However, there has also been a related body of work demonstrating that some central synapses can

respond to chronic drug applications in multiple ways. Depending upon the particular preparation or experimental condition, the synaptic response may also be governed through presynaptic changes in RRP size and/or transmitter release (Murthy et al., 2001; Burrone et al., 2002; Thiagarajan et al., 2005). These presynaptic changes do not have to be induced via network-wide perturbations. For example, silencing individual neurons with the inwardly rectifying potassium channel Kir2.1 after synapse formation causes a homeostatic increase of synaptic inputs (Burrone et al., 2002). But what exactly are the presynaptic events that control these responses? Recent results are shedding light on these phenomena.

Presynaptic calcium indicators

Presynaptic examinations have been illuminating in defining a role for Ca_V2 channels in HSP. For example, researchers utilizing a calcium reporter directly localized to synaptic vesicles (SyGCaMP2) demonstrated homeostatic changes in presynaptic calcium influx at rodent hippocampal neurons (Zhao et al., 2011). Synaptic boutons pretreated with TTX for 2 days show a marked increase in presynaptic calcium influx after action potential delivery (Zhao et al., 2011). By contrast, gabazine treatment to inhibit inhibitory $GABA_A$ receptors results in decreased presynaptic calcium influx (Zhao et al., 2011). These findings are strikingly reminiscent of those at the Drosophila NMJ (Müller and Davis, 2012). They are also consistent with microscopy data from cultured rodent cortical neurons and the aforementioned Drosophila NMJ, demonstrating that challenges to synaptic activity can result in profound homeostatic changes to components of the presynaptic release machinery (Lazarevic et al., 2011; Weyhersmüller et al., 2011).

Retrograde signaling: mTORC1, BDNF, and Ca_V2

Other results examining hippocampal cultures corroborate this notion. NBQX- or CNQX-mediated blockade of AMPA receptors in the absence of TTX induces two main homeostatic responses: (1) incorporation of new, GluA2-lacking AMPA receptors postsynaptically; and (2) a retrograde signaling process that results in increased presynaptic release properties, such as spontaneous miniature frequency (Thiagarajan et al., 2005; Gong et al., 2007). Similar to the Drosophila system, postsynaptic mammalian Target of Rapamycin Complex1 (mTORC1) drives this retrograde signaling process—albeit through release of Brain-Derived Neurotrophic Factor (BDNF), which is not found in Drosophila (Henry et al., 2012). Also resonant with the Drosophila NMJ, coincident application of a cocktail of N- and P/Q-type calcium channel blockers ω-conotoxin GVIA and ω-agatoxin IVA (CTx/ATx cocktail) completely abolishes the enhanced presynaptic activity induced by 3 h of CNQX exposure (Jakawich et al., 2010). This result supports the idea that presynaptic Ca_V2 function is required for this form of homeostatic plasticity.

Cyclin-dependent kinase 5 (CDK5) and calcineurin

Key components of $Ca_V2.2$ (N-type) regulation during HSP appear to be the cyclin-dependent kinase CDK5 and the α isoform of the phosphatase Calcineurin A (CNAα). Inhibition of CDK5 function in CA1-CA3 hippocampal cultures with the drug roscovitine results in enhanced action potential-evoked release and access to the resting synaptic vesicle pool for release (Kim and Ryan, 2010). Consistently, biochemical assays show that chronic silencing of synapses with TTX leads to a measurable decrease of CDK5 (Kim and Ryan, 2010). CDK5 exerts its control of synaptic activity by balancing an opposing function of CNAα (Kim and Ryan, 2010, 2013). The ability of either enzyme to exert control over action potential-driven exocytosis is reliant upon N-type calcium channels, as the N-type blocker ω-conotoxin GVIA occludes their effects (Kim and Ryan, 2013).

CA_V1 CHANNELS AND POSTSYNAPTIC HOMEOSTATIC SYNAPTIC PLASTICITY

In contrast to Ca_V2 channels, L-type Ca_V1 channels are localized to both presynaptic and postsynaptic structures in neurons, as well as non-neuronal excitable tissues—such as skeletal and cardiac muscle—where they are required for excitation-contraction coupling (Hell et al., 1993; Lipscombe et al., 2004; Obermair et al., 2004). Calcium entry though L-type channels activates a variety of downstream calcium-sensitive signaling cascades and gene regulation programs (Lipscombe et al., 2004). There are several sub-types of L-type Ca_V1 channels, classified by the α_1 subunit incorporated into the channel: $Ca_V1.1$, 1.2, 1.3, and 1.4 (mammalian α_1 subunit genes *CACNA1S*, *CACNA1C*, *CACNA1D*, and *CACNA1F* respectively). The kinetics of Ca_V1 activation can be fast (Lipscombe et al., 2004; Helton et al., 2005), however, it is generally thought that slow L-type deactivation allows sustained current, and thus, calcium signaling (McCobb and Beam, 1991; Perrier et al., 2002; Helton et al., 2005). Ca_V1-type calcium channels are sensitive to dihydropyridine antagonists such as nifedipine or nimodipine, or to phenylalkylamines like verapamil. Neuroscientists typically apply these drugs to electrophysiological preparations in order to assess the results of impaired Ca_V1 function. These drugs have been used to great effect to show that L-type channels are critical for the expression of a variety of forms of neuroplasticity (Murphy et al., 1991; Magee and Johnston, 1997; Hardingham et al., 1998; Weisskopf et al., 1999; Mermelstein et al., 2000; Brosenitsch and Katz, 2001; Dolmetsch et al., 2001; Lei et al., 2003; Pak and Sheng, 2003)—as well as forms of HSP, detailed below.

PIONEERING FINDINGS: CHRONIC BLOCKADE OF HIPPOCAMPAL NEURON ACTIVITY

In the same study that examined NBQX-induced blockade and the resulting incorporation of GluA2-lacking AMPA receptors in hippocampal cultures, it was reported that nifedipine application mimics NBQX blockade (Thiagarajan et al., 2005). This result suggests that loss of calcium influx through Ca_V1 channels could gate the mechanism of adding GluA1 homomeric (GluA2-lacking) AMPA receptors to the synapse. To test if Ca_V1 blockade and AMPA receptor blockade activate a shared mechanism, nifedipine and NBQX were applied concurrently. Dual drug application was indistinguishable from single drug application by biochemical and electrophysiological assays, consistent with a model in which AMPA blockade results in a loss of Ca_V1 activity (Thiagarajan et al., 2005). In turn, loss of Ca_V1 activity

results in a postsynaptic increase in GluA1 homomers as well as an increase in presynaptic release properties (Thiagarajan et al., 2005). But does Ca_V1-mediated plasticity occur exclusively after AMPA receptor blockade? No. For example, potentiation of postsynaptic miniature excitatory postsynaptic current (mEPSC) amplitudes also occurs when hippocampal neurons are silenced for 24 h with TTX and allowed to recover in TTX-free saline for a short time before recording (Sokolova and Mody, 2008). In this case, however, TTX/TTX washout-induced potentiation is *occluded* by nifedipine (Sokolova and Mody, 2008).

HOW CA_V1 SILENCING INDUCES HOMEOSTATIC SYNAPTIC PLASTICITY

What molecular mechanisms underlie silencing-induced metaplasticity downstream of Ca_V1? Recent research has implicated many classical calcium-sensitive cytoplasmic signaling molecules and processes. Through biochemistry and electrophysiology, one investigation demonstrated that downstream of CNQX-mediated AMPAR blockade (or blockade of Ca_V1), adenylate cyclase 1 activates nuclear transcription of new GluA1 subunits, which are then trafficked and incorporated as homomers (Gong et al., 2007). Additionally, a recent study demonstrated that in rat hippocampal neurons, the scaffolding molecule guanylate kinase-associated protein (GKAP) plays a key role in both scaling up synaptic activity (after TTX application) and scaling down of activity (after bicuculline) (Shin et al., 2012). It was found that blockade of Calcium/Calmodulin-dependent Kinase II (CaMKII) activity by the drug KN-93 impairs both the recruitment and the removal of GKAP at synapses. Further examination showed that the source of calcium that results in the activation of CaMKII is important. NMDAR blockade by 2-amino-5-phosphonopentanoic acid (APV) stops bicuculline-driven GKAP removal from synapses; by contrast, Ca_V1 impairment by nimodipine stops TTX-induced GKAP enhancement at synapses (Shin et al., 2012).

Chronic neuronal silencing not only enhances the abundance of excitatory neurotransmitter receptors in a homeostatic fashion, but it can also decrease the abundance of inhibitory receptors at GABAergic synapses—consistent with network stability working through a balance of excitatory and inhibitory connections (Kilman et al., 2002; Swanwick et al., 2006; Saliba et al., 2007). Electrophysiological experiments have revealed a role for Ca_V1 in this homeostatic process too. For example, in hippocampal cultures, 24 h of nifedipine application reduces GABAergic synaptic transmission, likely due to an increased turnover and a decreased insertion of $GABA_A$ receptors (Saliba et al., 2009).

CA_V1 INTEGRATES RESPONSES TO CHRONIC ACTIVATION

If loss of calcium influx through postsynaptic Ca_V1 channels mediates homeostatic forms of potentiation (**Figure 1A**), could increased calcium influx through Ca_V1 mediate homeostatic forms of depression (**Figure 1B**)? Yes—one elegant analysis showed that this is likely true and went further to demonstrate that homeostatic signaling events governed by Ca_V1 occur not just on the level of entire networks, but also on the level of single cells (Goold and Nicoll, 2010). By taking advantage of optogenetics, the authors of this study excited individual Channelrhodopsin 2 (ChR2)-expressing CA1 pyramidal neurons for 24 h. In response to this excitatory stimulation, there is a homeostatic downregulation of both NMDAR and AMPAR-mediated responses postsynaptically. This compensatory, homeostatic depression works via L-type calcium channels, as nifedipine occludes the effect (Goold and Nicoll, 2010).

CELLULAR MECHANISMS AFTER CHRONIC ACTIVATION

What are the cellular mechanisms that drive homeostatic downscaling after chronic activation? This question has recently been reviewed (Siddoway et al., 2014). Here, we cover data integrating Ca_V1 with homeostatic downscaling. In the ChR2-expressing CA1 neurons, this process appears to require Calcium/Calmodulin-dependent protein kinase kinase (CaMKK)—the CaMKK inhibitor STO-609 blocks homeostatic depression (Goold and Nicoll, 2010). This process also requires CaM kinase 4 (CaMK4), as a CaMK4 dominant negative construct also occludes depression (Goold and Nicoll, 2010).

Similar manipulations have uncovered additional calcium-responsive integrators of HSP. In an analysis employing cultured mouse cortical neurons, it was found that chronic bicuculline application causes phosphorylation of the protein phosphatase 1 (PP1) inhibitor I-2. In turn, I-2 phosphorylation is required for appropriate AMPAR trafficking and homeostatic downregulation of synaptic function (Siddoway et al., 2013). L-type calcium channels and calmodulin activity are required for I-2 phosphorylation. Addition of nimodipine or the calmodulin antagonist W7 both block increases in I-2 phosphorylation provoked by bicuculline (Siddoway et al., 2013).

Another example crops up in neocortical neurons, where prolonged hyperactivity induced by gabazine induces increases of production of vesicular glutamate transporter (VGLUT2) and neuronal activity-regulated pentraxin (Narp; Doyle et al., 2010). These increases are hypothesized to be homeostatic in nature because they may lead to increased activation of GABAergic inhibitory feedback neurons (Rutherford et al., 1997; Turrigiano and Nelson, 2004). This form of excitation-transcription coupling is dependent upon Ca_V1 channels—VGLUT2/Narp induction by gabazine is blocked by nifedipine and verapamil application (Doyle et al., 2010). What acts downstream of Ca_V1? More classical calcium signaling molecules: VGLUT2/Narp induction is blocked by CaMK antagonists KN-62 and KN-93, as well as mitogen-activated protein kinase (MAPK) antagonists PD98059 and U0126 (Doyle et al., 2010).

CA_V CHANNELS AND THE HOMEOSTATIC GROWTH AND PRUNING OF SYNAPSES

If the end goal of homeostatic plasticity is to keep synapses and circuits functioning within normal physiological ranges, then a logical way to accomplish this task is to add or prune synaptic connections and couple development with activity. The relationship between synaptic growth, developmental plasticity, neurotransmission, and HSP is not entirely understood. The coupling of these processes appears to depend upon the particular system or manipulation examined. With Hebbian forms of plasticity like LTP or LTD, one observes a clear correlation between the growth (potentiation) or shrinkage (depression) of dendritic spines in central synapses (Matsuzaki et al., 2004; Zhou et al., 2004; Tada and Sheng, 2006; Lisman et al., 2012). This is not always the

case with homeostatic plasticity. Above we have considered several examples of long-lasting homeostatic mechanisms gated by VGCCs. Many of these involve changes to presynaptic release probability or to postsynaptic neurotransmitter receptor composition, yet do not involve gross morphological changes to synaptic architecture.

Nevertheless, it would be wrong to claim that synapse/circuit function is completely divorced from developmental forms of homeostatic plasticity. The visual systems of mammals and fruit flies offer elegant examples in which developmental homeostatic programs compensate for visual or activity depravation (Mrsic-Flogel et al., 2007; Yuan et al., 2011; Whitt et al., 2014). In the realm of VGCCs and downstream calcium signaling, there are data that suggest direct or indirect control synaptic growth processes that could influence homeostasis. We consider a few examples from both invertebrate and vertebrate systems.

DROSOPHILA NEUROMUSCULAR JUNCTION (NMJ)

Do long-lasting disruptions of neurotransmission cause synapses to assume alternate, homeostatic developmental programs? Conversely, do developmental alterations result in aberrant neurophysiology? At the Drosophila NMJ, much data suggest that developmental changes are not necessarily coupled to alterations of neurotransmission. A recent study examining evolutionarily diverged species of Drosophila showed that wide variations of "wild-type" synaptic growth—including marked differences in bouton number and branching at the NMJ—redound to indistinguishable physiology (Campbell and Ganetzky, 2012, 2013). These data and the data of others suggest that the properties of synapse growth and function can be uncoupled at insect NMJs.

Concerning Ca_V channels, two studies reported that partial *cac* loss-of-function mutations affect synaptic growth by causing a mild decrease in the number of synaptic boutons that are formed (Rieckhof et al., 2003; Xing et al., 2005). Another has shown that null mutations in the $\alpha_2\delta$ subunit gene of Ca_V2 fail to develop NMJ boutons in embryos (Kurshan et al., 2009). Finally, in a study examining synaptic overgrowth caused by potassium channel mutations, it was found that both postsynaptic L-type Ca_V1 channels (α_1 subunit encoded by Drosophila *Dmca1D*) and presynaptic Ca_V2/Cac channels participate in enabling the overgrowth, at the respective stages of bouton budding (DmCa1D) and maturation (Cac) (Lee and Wu, 2010). It is unclear whether the roles of VGCCs during overgrowth are mechanistically the same as during normal developmental growth.

In all, the aggregate data show that the activity of Drosophila Ca_V channels can positively influence NMJ maturation and growth (**Figure 2A**). In the sense that synapse growth is developmentally coincident with larval growth, this could be considered to be a homeostatic function. However, NMJ bouton developmental phenotypes can clearly be uncoupled from neurotransmission phenotypes. This point highlights the importance of examining both development and electrophysiological responses in this model synapse.

CAENORHABDITIS ELEGANS SYNAPSE DEVELOPMENT

Ca_V2 and Ca_V1 channels govern a number of behaviors studied in nematode worms, such as coordination of normal movement and egg laying (Brenner, 1974; Trent et al., 1983; Schafer and Kenyon, 1995; Lee et al., 1997). In *C. elegans*, the Ca_V2 α_1 homolog is called UNC-2 (Schafer and Kenyon, 1995), and the Ca_V1 α_1 homolog is called EGL-19 (Lee et al., 1997). *C. elegans* is an excellent system to study Ca_V functions because there are strong loss-of-function mutations in the genes encoding these pore-forming subunits, as well as mutations in the genes encoding auxiliary subunits, like the $\alpha_2\delta$ homolog, UNC-36 (**Figure 2B**). Interestingly, UNC-36 has been shown to be important not only for UNC-2/Ca_V2 localization and function as would be expected for $\alpha_2\delta$ (Saheki and Bargmann, 2009), but it also modulates the activation and conductance of EGL-19/Ca_V1-mediated calcium currents (Frøkjaer-Jensen et al., 2006; Lainé et al., 2011).

Throughout *C. elegans* larval development, the density of synapses (the number of synapses per unit length) containing GLR-1 glutamate receptors in the ventral nerve cord (VNC) remains at a set point level, even though absolute synapse number increases dramatically over time (Rongo and Kaplan, 1999). The tight coupling between GLR-1 synapse formation and VNC growth is likely homeostatic, and it is reminiscent of the coupling between organism growth and synapse formation seen at the Drosophila and mammalian NMJs. In *C. elegans*, mutations in the *unc-43* gene—which encodes CaMKII—significantly reduce synaptic density of transgenic GLR-1::GFP protein in the VNCs of adult worms (Rongo and Kaplan, 1999). This indicates an uncoupling between growth and synapse development. This result prompted an investigation into the source of calcium upstream of UNC-43/CaMKII; it was found that *unc-2* and *egl-19* mutations also significantly reduce synapse density (Rongo and Kaplan, 1999). However, in addition to synapse density, GLR-1::GFP puncta intensity was examined in a follow-up study—and here it was found that *unc-2* loss results in a compensatory *increase* in glutamate receptor intensity (Grunwald et al., 2004). Taken together with data from other systems, these *C. elegans* data are consistent with an ancient role in synapse development for both P/Q- and L-type channels.

The importance of CaMKII and Ca_V function carries over to the NMJs of *C. elegans*. A recent study demonstrated that mutations in *unc-43*, *unc-2*, or *unc-36* all have altered NMJ morphology (Caylor et al., 2013). A close examination of the NMJs during the L4 larval stage revealed that wild-type NMJs add new boutons in a dynamic process, evidenced by enlarged or elongated puncta of a GFP-tagged Synaptobrevin marker. By contrast, in *unc-2* mutants, this process is muted (**Figure 2B**; Caylor et al., 2013).

STRIATAL MEDIUM SPINY NEURONS

Striatal medium spiny neurons (MSNs) offer a vertebrate model of homeostatic control of synaptic growth gated by VGCCs. In MSNs, increased striatal dopamine causes an increase in MSN spine density (Kim et al., 2009). By contrast, decreased dopamine levels (as in Parkinson's Disease) result in a marked pruning of the MSN spine density, but also a decrease of glutamatergic synapses onto D2 dopamine receptor (D2R)-expressing MSNs (Day et al., 2006; Deutch et al., 2007). Elimination of D2R synapses enhances MSN excitability (Shen et al., 2008). Thus the pruning of D2R-containing synapses likely represents a homeostatic response to

the lack of dopamine. Follow-up work employing a combination of potassium-induced membrane depolarization and nimodipine blockage of L-type calcium channels demonstrates that MSN synaptic reduction is dependent upon $Ca_V1.2$ function. By contrast, the L-type channel agonist Bay K8644 enhances the effects of membrane depolarization (Tian et al., 2010). Downstream of calcium entry through $Ca_V1.2$, there is Calcineurin-mediated activation of Mef2 transcription factor activity (Tian et al., 2010). Consistently, Mef2 has been reported to regulate developmental synaptic remodeling by controlling the expression of a variety of target genes (Flavell et al., 2006, 2008; Fiore et al., 2009; Ye et al., 2013).

POSSIBLE LINKS BETWEEN CA_V CHANNELOPATHIES AND HOMEOSTATIC PLASTICITY

Ion channel disorders (channelopathies) can have debilitating manifestations (Kullmann, 2010; Ryan and Ptacek, 2010). The fact that stable synapse function depends on homeostatic signaling leads to a logical question: do some channelopathies result from impaired homeostatic systems? The answer is not 100% clear for any disorder. However, several channelopathies—such as forms of epilepsy and migraine—display a possible hallmark of impaired HSP: long periods of neuronal stability followed by sudden, episodic attacks. A series of reviews recently surveyed many calcium channelopathies, including those caused by mutations in subunit genes for Ca_V1-, Ca_V2-, and Ca_V3-type channels (Liao and Soong, 2010; Pietrobon, 2010a; Striessnig et al., 2010; Zamponi et al., 2010; Cain and Snutch, 2011). Another recent review examined compelling connections between HSP and neurological disorders (Wondolowski and Dickman, 2013). Knowing what we know about VGCCs and HSP, we may draw speculative links between some VGCC channelopathies and homeostatic plasticity. Here we consider a subset of them, including episodic forms of migraine, ataxia, myasthenia, epilepsy, and paralysis.

$CA_V2.1$ CHANNELOPATHIES: MIGRAINE, ATAXIA, AND MYASTHENIA

Two $Ca_V2.1$ channelopathies—familial hemiplegic migraine type 1 (FHM1) and episodic ataxia type 2 (EA2)—result from mutations (gain- and loss-of-function, respectively) in human *CACNA1A*, which encodes the α_1 subunit of presynaptic $Ca_V2.1$-type calcium channels (Ophoff et al., 1996; Pietrobon, 2010a). A third disorder—spinocerebellar ataxia type 6 (SCA6)—results from poly-glutamine (polyQ) expansion in the *CACNA1A* gene product (Zhuchenko et al., 1997). There is some question about whether SCA6 is more properly classified as polyQ expansion disorder rather than a channelopathy. Here we focus on FHM1 and EA2.

Migraine is the most common neurological disorder—about 12% of the population suffers from it, with a high lifetime incidence rate for women (Barrett et al., 2008; Stewart et al., 2008; NINDS/NIH, 2009). FHM1 is a rare subtype; it is an inherited $Ca_V2.1$ channelopathy that causes migraine with an accompanying aura (Ophoff et al., 1996; Pietrobon, 2010a,b). While some gain-of-function *CACNA1A* mutations cause only FHM1, others can lead to additional maladies, such as epileptic attacks (Kors et al., 2001; Pietrobon, 2010a,b; van den Maagdenberg et al., 2010). Cortical Spreading Depression (CSD) is associated with aura and FHM1 migraines. CSD is defined as a wave of depolarization of neural cells along the cerebral cortex, followed a prolonged period of inactivity (Charles and Baca, 2013). CSD can be monitored in real time with Blood-Oxygen-Level Dependent functional Magnetic Resonance Imaging (BOLD-fMRI). It is unclear whether CSD is coincident with types of migraine other than FHM. However, the fact that a sudden wave of cortical depolarization is tightly coupled to a form of headache is consistent with the idea that homeostatic mechanisms keep neuronal functions within normal physiological ranges—and more importantly, that these mechanisms could be impaired in FHM1 migraine sufferers. There exist knock-in mouse models of FHM1 (van den Maagdenberg et al., 2004, 2010). These knock-in models have been a wonderful resource to deduce P/Q-channel properties of FHM1-inducing amino acid substitutions and to establish that the substitutions do represent gains of channel function. Animal models may also prove to be valuable in examining whether forms of migraine correlate with an underlying disruption in HSP.

In contrast to FHM1, EA2 is caused by loss-of-function mutations in *CACNA1A* (Ophoff et al., 1996; Pietrobon, 2010a). Most of the known EA2 mutations are dominant missense mutations that affect the trafficking of channel subunits, but some cause *CACNA1A* splicing defects (Pietrobon, 2010a). Like most forms of ataxia, EA2 is marked by sudden attacks of uncoordinated movement. The episodic nature of these attacks again suggests a possible impairment in homeostatic plasticity—speculation made all the more intriguing given that Drosophila *cac* loss-of-function mutations impair homeostatic plasticity at the NMJ (Frank et al., 2006, 2009; Müller and Davis, 2012). Again, several rodent EA2 models exist, including spontaneous loss-of-function *CACNA1A* mutations in *tottering*, *leaner*, *rolling Nagoya*, and *rocker* mice—as well as mice lacking functional P/Q channels altogether (Pietrobon, 2005, 2010a; Miki et al., 2008; Plomp et al., 2009). Homozygous *CACNA1A* $^{-/-}$ loss-of-function mutant mice display severe forms of ataxia. It may be fruitful to probe these mice (or neuronal cultures derived from them) to check if homeostatic plasticity is disrupted.

Forms of myasthenia cause muscle weakness. For example, myasthenia gravis is an autoimmune disorder that directly affects the NMJ. Antibodies formed against acetylcholine receptors impair muscle function. This occurs despite the NMJ's apparent attempts to correct the problem via homeostatic increases in presynaptic quantal content—an observation from both human myasthenic muscle and rodent models of myasthenia gravis (Cull-Candy et al., 1980; Plomp et al., 1992, 1994, 1995). A separate, rare form of myasthenia is Lambert-Eaton myasthenic syndrome (LEMS; Marion et al., 1984; Pascuzzi, 2002; Mareska and Gutmann, 2004). Like myasthenia gravis, LEMS is an autoimmune disorder, but it is also a channelopathy because autoantibodies are formed against $Ca_V2.1$-type calcium channels (Motomura et al., 2000). Mammalian NMJs appear to be endowed with homeostatic coping mechanisms, much like insect NMJs. However, in the case of LEMS, interfering with the NMJ's ability to generate sufficient presynaptic calcium influx may cause sufficient stress to cause myasthenic symptoms.

CA_V MUTATIONS AND EPILEPSY

The term epilepsy encompasses a range of seizure-associated maladies. Epilepsy is broadly defined by multiple seizure events in a single individual (NINDS/NIH, 2004). It is likely the most frequently invoked neurological disorder that is hypothesized to be triggered or facilitated by defects in neuronal homeostatic signaling. This is logical because epilepsies are marked by an underlying instability of neuronal function.

In addition to ataxia, $Ca_V2.1$-deficient "EA2" mice experience absence seizures—i.e., brief events that are marked by an abrupt arrest in activity, followed by a return to normal activity (Noebels and Sidman, 1979; Jun et al., 1999). Mice that have loss-of-function mutations in genes encoding $\alpha_2\delta$ and γ_2 calcium channel subunits and a reduction in $Ca_V2.1$ channel activity also display absence seizures (Letts et al., 1998; Barclay et al., 2001; Letts et al., 2003). In humans with heterozygous EA2-causing mutations, absence seizures are not common, but there are instances in which they are present (Zamponi et al., 2010).

$Ca_V3.1$ and $Ca_V3.2$ T-type calcium channels contribute to a spectrum of polygenic epilepsies. Mutations in the $Ca_V3.1$ α_1 subunit gene *CACNA1G* have been implicated in idiopathic generalized epilepsy (IGE) susceptibility (Singh et al., 2007). Likewise, mutations in the $Ca_V3.2$ α_1 subunit gene *CACNA1H* have been implicated in childhood absence epilepsy (CAE) susceptibility (Chen et al., 2003; Vitko et al., 2005). Canonically, T-type calcium channels are responsible for low-threshold spiking activity in thalamic neurons (Llinas, 1988). As T-type calcium channels open, there is a significant increase in local intracellular calcium levels (Errington et al., 2010); thus, one can easily hypothesize that proper regulation of T-type currents is critical to neuronal stability. In several rodent models of absence epilepsy, there is a robust increase in T-type calcium currents (Tsakiridou et al., 1995; Talley et al., 2000; Broicher et al., 2008). This is true not only for rodent models in which $Ca_V3.1$ currents themselves are high (Ernst et al., 2009), but also for cases in which a loss of P/Q-channel activity results in an increase in T-channel activity (Zhang et al., 2002). The latter example may illustrate a hierarchy of homeostatic signals, in which overall calcium currents are maintained at the expense of neuronal stability. A similar type of model has been proposed for potassium currents and homeostasis at the Drosophila NMJ (Bergquist et al., 2010).

$CA_V1.1$ AND HYPOKALEMIC PERIODIC PARALYSIS

Starting around adolescence, individuals who suffer from hypokalemic periodic paralysis (HPP) experience intermittent episodes of muscle weakness and paralysis that last on the order of 3–24 h in length. The attacks often occur upon waking or after high levels of carbohydrate consumption (USNLM/NIH, 2011). The episodic nature of these attacks and the mild challenges that trigger them could suggest defective homeostasis in muscle cells. Familial HPP type 1 is caused by missense mutations in *CACNA1S*, which encodes the α_1 subunit of $Ca_V1.1$, and most of the mutations alter arginine residues in the S4 voltage sensor (Sipos et al., 1995; Morrill and Cannon, 1999). Further experiments exploiting muscle fibers from HPP1 patients have suggested that these arginine amino acid substitutions induce cation gating pore currents and that HPP1 muscle has a higher concentration of sodium (Jurkat-Rott et al., 2009).

CONCLUDING COMMENTS

Understanding how VGCCs help to execute homeostatic forms of neuroplasticity represents a new area of research. It offers a fresh way to consider why VGCCs are regulated in the ways that they are. In healthy neurons, it is logical that calcium channels are co-opted to gate homeostatic signaling processes. Presynaptically, neurotransmitter release is dependent upon calcium influx or local domains of high calcium concentration. Postsynaptically, many signaling processes are dependent upon calcium that enters the cell through receptor complexes and L-type calcium channels. Neurons, synapses, and circuits across metazoan nervous systems are endowed with powerful homeostatic set points of activity, and small adjustments to calcium channel activity parameters could help fine tune synaptic outputs in a variety of ways.

An important frontier for research will be to understand how Ca_V channels interact with intracellular signaling mechanisms and with the activities of other ion channels to control homeostatic set points of excitable cells. What signaling processes directly modulate Ca_V2 during homeostatic modulations of neurotransmitter release? What postsynaptic cascades are activated or suppressed downstream of calcium influx through Ca_V1? We have excellent clues at this date from diverse experimental systems reviewed here. Concerning HSP, it will be interesting to see which modes of regulation prove to be universal and which ones prove to be synapse- or organism-specific. With that knowledge in hand, it may be possible to better understand and address numerous phenomena, including Ca_V channelopathies.

ACKNOWLEDGMENTS

Many thanks to members of the Frank laboratory for helpful discussions. This work was supported in part by NIH grant NS062738, as well as funds from the Department of Anatomy and Cell Biology at the University of Iowa Carver College of Medicine, and a Carver Trust Medical Research Initiative Grant.

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Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 09 December 2013; accepted: 28 January 2014; published online: 14 February 2014.

*Citation: Frank CA (2014) How voltage-gated calcium channels gate forms of homeostatic synaptic plasticity. Front. Cell. Neurosci. **8**:40. doi: 10.3389/fncel.2014.00040*

This article was submitted to the journal Frontiers in Cellular Neuroscience.

frontiers in Cellular Neuroscience

ORIGINAL RESEARCH
published: 09 June 2015
doi: 10.3389/fncel.2015.00214

Facial stimulation induces long-term depression at cerebellar molecular layer interneuron–Purkinje cell synapses *in vivo* in mice

Yan-Hua Bing[1,2†], *Mao-Cheng Wu*[1,3†], *Chun-Ping Chu*[1*] *and De-Lai Qiu*[1,2*]

[1] *Cellular Function Research Center, Yanbian University, Yanji, Jilin Province, China,* [2] *Department of Physiology and Pathophysiology, College of Medicine, Yanbian University, Yanji, Jilin Province, China,* [3] *Department of Osteology, Affiliated Hospital of Yanbian University, Yanji, Jilin Province, China*

Cerebellar long-term synaptic plasticity has been proposed to provide a cellular mechanism for motor learning. Numerous studies have demonstrated the induction and mechanisms of synaptic plasticity at parallel fiber–Purkinje cell (PF–PC), parallel fiber–molecular layer interneurons (PF–MLI) and mossy fiber–granule cell (MF–GC) synapses, but no study has investigated sensory stimulation-evoked synaptic plasticity at MLI–PC synapses in the cerebellar cortex of living animals. We studied the expression and mechanism of MLI–PC GABAergic synaptic plasticity induced by a train of facial stimulation in urethane-anesthetized mice by cell-attached recordings and pharmacological methods. We found that 1 Hz, but not a 2 Hz or 4 Hz, facial stimulation induced a long-term depression (LTD) of GABAergic transmission at MLI–PC synapses, which was accompanied with a decrease in the stimulation-evoked pause of spike firing in PCs, but did not induce a significant change in the properties of the sensory-evoked spike events of MLIs. The MLI–PC GABAergic LTD could be prevented by blocking cannabinoid type 1 (CB1) receptors, and could be pharmacologically induced by a CB1 receptor agonist. Additionally, 1 Hz facial stimulation delivered in the presence of a metabotropic glutamate receptor 1 (mGluR1) antagonist, JNJ16259685, still induced the MLI–PC GABAergic LTD, whereas blocking N-methyl-D-aspartate (NMDA) receptors during 1 Hz facial stimulation abolished the expression of MLI–PC GABAergic LTD. These results indicate that sensory stimulation can induce an endocannabinoid (eCB)-dependent LTD of GABAergic transmission at MLI–PC synapses via activation of NMDA receptors in cerebellar cortical Crus II *in vivo* in mice. Our results suggest that the sensory stimulation-evoked MLI–PC GABAergic synaptic plasticity may play a critical role in motor learning in animals.

Keywords: cerebellar Purkinje cell, molecular layer interneuron, sensory stimulation, plasticity, NMDA receptor, endocannabinoids receptor, *in vivo* cell-attached recording

OPEN ACCESS

Edited by:
Jaichandar Subramanian, Massachusetts Institute of Technology, USA

Reviewed by:
Christian Hansel, Erasmus Medical Center, Netherlands
Ying Shen, Zhejiang University, China

***Correspondence:**
Chun-Ping Chu and De-Lai Qiu, Cellular Function Research Center, YanBian University, 977, GongYuan Road, Yanji City, 133002, Jilin Province, China
cpchu@ybu.edu.cn; dlqiu@ybu.edu.cn

[†] *These authors have contributed equally to this work.*

Received: *01 April 2015*
Accepted: *20 May 2015*
Published: *09 June 2015*

Citation:
Bing Y-H, Wu M-C, Chu C-P and Qiu D-L (2015) Facial stimulation induces long-term depression at cerebellar molecular layer interneuron–Purkinje cell synapses in vivo in mice. Front. Cell. Neurosci. 9:214. doi: 10.3389/fncel.2015.00214

Introduction

Synaptic plasticity is a modification of synaptic strength, which is important to the formation and stability of neuronal circuits. Cerebellar long-term synaptic plasticity can be induced at

parallel fiber–Purkinje cell (PF–PC), parallel fiber–molecular layer interneuron (PF–MLI), mossy fiber–granule cell (MF–GC) and MLI–PC synapses under *in vitro* conditions, and has been proposed as a cellular mechanism for motor learning (Grasselli and Hansel, 2014).

The cerebellar cortex includes the molecular layer (ML), Purkinje cell layer (PCL) and granule cell layer (GCL), which are mainly populated by molecular interneurons (MLI), Purkinje cells (PC), and granule cells (GC) and Golgi cells, respectively (Eccles et al., 1967). Synaptic plasticity at PF–PC, PF–MLI, and MF–GC synapses has been widely studied in cerebellar slices (Ito and Kano, 1982; Qiu and Knöpfel, 2007, 2009; D'Errico et al., 2009; Piochon et al., 2010; Garrido et al., 2013; D'Angelo, 2014; van Beugen et al., 2014; Yamazaki et al., 2015) and in living animals (Roggeri et al., 2008; Márquez-Ruiz and Cheron, 2012; Chu et al., 2014). At MLI–PC synapses, three types of plasticity have been induced by postsynaptic depolarization under *in vitro* conditions; depolarization-induced potentiation of inhibition (DPI), depolarization-induced suppression of inhibition (DSI) and rebound potentiation (RP; Hirano, 2013). DSI is a type of short-lasting suppression in presynaptic GABA release mediated by endocannabinoids (eCB), which are released from a PC and bind to presynaptic CB1 receptors (Llano et al., 1991a; Yoshida et al., 2002). In contrast, DPI is a long-term potentiation (LTP) of presynaptic GABA release mediated by glutamate release from a postsynaptic PC, which then binds to presynaptic NMDA receptors (Duguid and Smart, 2004). RP occurs postsynaptically and lasts longer; it is induced by a Ca^{2+}-dependent upregulation of $GABA_A$ receptor activity on PCs (Kano et al., 1992, 1996; Kawaguchi and Hirano, 2000, 2007; Tanaka et al., 2013; Hirano and Kawaguchi, 2014). Moreover, repetitive stimulation of CFs can induce GABAergic transmission LTP in PCs (Kano et al., 1996; Kawaguchi and Hirano, 2002). The GABAergic transmission LTP at MLI–PC synapses requires an enhanced postsynaptic Ca^{2+} transient in PCs through voltage-gated Ca^{2+} channels and inositol 1, 4, 5-triphophate (IP3)-mediated Ca^{2+} release from internal stores. The increased Ca^{2+} transient activates calmodulin-dependent protein kinase II (CaMKII), which in turn regulates GABAergic transmission at MLI–PC synapses via $GABA_A$ receptors (Kano et al., 1992, 1996; Kawaguchi and Hirano, 2007).

Similar to the synaptic plasticity at PF–PC, PF–MLI, and MF–GC synapses, MLI–PC synaptic plasticity may also be related to cerebellar motor learning (Hirano and Kawaguchi, 2014). PC-specific deletion of $GABA_A$ receptor $\gamma 2$ subunits, which removes all inhibition of PCs, affects both phase reversal learning, and gain and phase consolidation of the vestibulo-ocular reflex (Wulff et al., 2009; Seja et al., 2012). Moreover, CaMKII deletion in PCs, may also affect the MLI–PC synapse indirectly and influence both gain increase and decrease in vestibulo-ocular reflex learning (Hansel et al., 2006; Schonewille et al., 2010). Therefore, MLI–PC synaptic plasticity might be related to the coding of cerebellar cortical information and motor learning.

Collectively, MLI–PC synaptic plasticity has been studied in cerebellar slices, but the mechanisms of sensory stimulation-evoked synaptic plasticity at MLI–PC synapses in the cerebellar cortex of living animals are currently unknown. Therefore, we studied the mechanism of MLI–PC GABAergic synaptic plasticity induced by a train of facial stimulation in urethane-anesthetized mice by physiological and pharmacological methods. Our results showed that 1 Hz, but not 2 Hz or 4 Hz, facial stimulation induced GABAergic transmission LTD at MLI–PC synapses, accompanied with a decrease in the sensory-evoked pause of spike firing. The MLI–PC GABAergic LTD could be prevented by blocking CB1 receptors, and could be pharmacologically induced by a CB1 receptor agonist. MLI–PC GABAergic LTD was not blocked by an mGluR1 antagonist, but was abolished by blockade of NMDA receptors during 1 Hz facial stimulation. These results indicate that sensory stimulation induces an eCB-dependent LTD of GABAergic transmission at MLI–PC synapses via activation of NMDA receptors *in vivo* in mice.

Materials and Methods

Anesthesia and Surgical Procedures

The anesthesia and surgical procedures have been described previously (Chu et al., 2011a,b). The experimental procedures were approved by the Animal Care and Use Committee of Jilin University and were in accordance with the animal welfare guidelines of the U.S. National Institutes of Health. The permit number is SYXK (Ji) 2007-0011. Adult (6–8-week-old) HA/ICR mice were anesthetized with urethane (1.3 g/kg body weight, i.p.). Mice were tracheotomized to avoid respiratory obstruction. On a custom-made stereotaxic frame, soft tissue was retracted to gain access to the dorsal portion of the occipital bone. A watertight chamber was created and a 1–1.5-mm craniotomy was drilled to expose the cerebellar surface corresponding to Crus II. The brain surface was constantly superfused with oxygenated artificial cerebrospinal fluid (ACSF: 125 mM NaCl, 3 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 1 mM NaH_2PO_4, 25 mM $NaHCO_3$, and 10 mM D-glucose) with a peristaltic pump (Gilson Minipulse 3; Villiers, Le Bel, France) at 0.4 ml/min. Rectal temperature was monitored and maintained at 37.0 $\pm$ 0.2°C using body temperature equipment.

Cell-Attached Recording and Facial Stimulation

Cell-attached recordings from PCs were performed with an Axopatch-200B amplifier (Molecular Devices, Foster City, CA, USA). The signals of PC cell-attached recordings were acquired through a Digidata 1440 series analog-to-digital interface on a personal computer using Clampex 10.3 software (Molecular Devices). Patch pipettes were made with a puller (PB-10; Narishige, Tokyo, Japan) from thick-wall borosilicate glass (GD-1.5; Narishige). Recording electrodes were filled with ACSF, with resistances of 3–5 MΩ. The cell-attached recordings from PCs were performed at depths of 150–200 μm under the pia mater membrane, and were identified by regular spontaneous simple spikes (SSs) accompanied with irregular

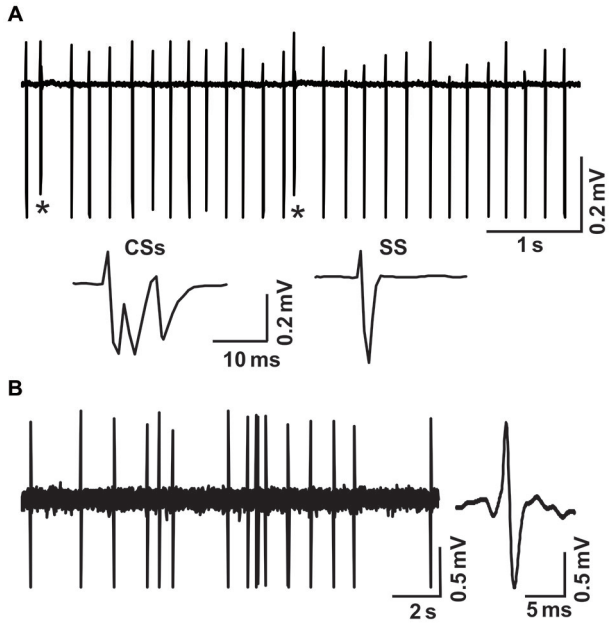

FIGURE 1 | Cell-attached recordings showing the characterization of cerebellar cortical PCs and MLIs *in vivo* in mice. (A) Upper, representative cell-attached recording traces showing the spontaneous activity of a cerebellar PC. Lower, enlarged traces of CSs and a simple spike (SS). CSs are indicated by*. **(B)** Left, representative cell-attached recording traces showing the spontaneous activity of a cerebellar MLI. Right, enlarged traces of spike firing.

complex spikes. The MLIs were roughly identified by irregularly spontaneous spike activity and the depth of the recording site under the cell-attached recording condition (Chu et al., 2012).

Facial stimulation was performed by air-puff (10 ms, 60 psi) of the ipsilateral whisker pad through a 12-gauge stainless steel tube connected with a pressurized injection system (Picospritzer® III; Parker Hannifin Co., Pine Brook, NJ, USA). The air-puff stimulations were controlled by a personal computer, and were synchronized with the electrophysiological recordings and delivered at 0.05 Hz via a Master 8 controller (A.M.P.I., Jerusalem, Israel) and Clampex 10.3 software. The facial stimulation-evoked MLI–PC synaptic response has been demonstrated in our previous studies (Chu et al., 2011a,b); the response is a sequence of negative components (N1) followed by a positive component (P1) accompanied with a pause of SS firing (**Figure 1A**). For the induction of MLI–PC synaptic plasticity, 240 pulses of air-puff stimulation (10 ms, 60 psi) were delivered at 1 Hz, 2 Hz, and 4 Hz. The induction stimulation was delivered 10 min after the recording became stable.

Chemicals

The reagents used were D-aminophosphonovaleric acid (D-APV), the NMDA receptor antagonist; N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2, 4-di-chlorophenyl)-4-methyl-1H-pyrazole -3-carboxamide (AM251), for blocking endocannabinoid CB1 receptors; (3, 4-dihydro-2H-pyrano [2, 3-b]quinolin-7-yl)-(cis-4-methoxycyclohexyl)-methanone [JNJ16259685 (JNJ)], a group 1 metabotropic glutamate receptor antagonist; and (R)-(+)-[2, 3-dihydro-5-methyl-3-(4-morpholinylmethyl) pyrrolo[1, 2, 3-de]-1, 4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate (WIN55212-2), a CB1 receptor agonist. All chemicals were purchased from Sigma-Aldrich (Shanghai, China). The drugs were dissolved in ACSF, and applied directly onto the cerebellar surface by a peristaltic pump (0.5 ml/min).

Data Analysis

The electrophysiological data were analyzed using Clampfit 10.3 software (Molecular Devices, Foster City, CA, USA). Values are expressed as the mean ± SEM. ANOVA (posthoc multiple comparison; SPSS software) was used to determine the level of statistical significance among groups of data. *P*-values below 0.05 were considered statistically significant.

Results

Facial Stimulation (1 Hz) Induces GABAergic Transmission LTD at PCs in the Mouse Cerebellar Cortex

Under cell-attached recording conditions, PCs were identified by the presence of regular spontaneous SS firing activity accompanied with complex spikes. These PCs responded to air-puff stimulation (10 ms; 60 psi), which was expressed as a sequence of a negative component (N1) followed by a positive component (P1), accompanied by a pause in SS firing (**Figure 2A**). According to our previous studies (Chu et al., 2011a), N1 is a component of the PF volley, whereas P1 is sensitive to $GABA_A$ receptor antagonist, which identifies it as MLI–PC GABAergic synaptic transmission onto the PC. Based on the frequency properties of PCs (Bing et al., 2015b), we first examined whether MLI–PC GABAergic synaptic plasticity could be evoked by 1 Hz facial stimulation (240 pulses). This repetitive stimulation produced a persistent depression of MLI–PC GABAergic synaptic transmission, which was expressed as a decrease in P1 amplitude for over 50 min (**Figure 2A**). The normalized amplitude of P1 was decreased to 73.6 ± 12.4% of baseline for 40–50 min after 1 Hz facial stimulation ($P < 0.05$, $n = 7$, **Figure 1B**). In contrast, the normalized amplitude of P1 was 102.2 ± 10.3% of baseline at 40–50 min under control conditions ($P > 0.05$, $n = 8$, **Figure 2B**). Additionally, the normalized amplitude of N1 at 40–50 min after 1 Hz facial stimulation was 99.6 ± 25.3% of baseline, which did not change significantly after the repetitive stimulation ($P > 0.05$, $n = 8$, data not shown). These results indicated that 1 Hz facial stimulation induced GABAergic transmission LTD at PCs in the mouse cerebellar cortex.

Next, we examined whether the LTD of GABAergic transmission at PCs could be induced by 2 Hz and 4 Hz facial stimulation. As shown in **Figure 3**, the normalized amplitude of P1 at 40–50 min after 1 Hz facial stimulation was decreased

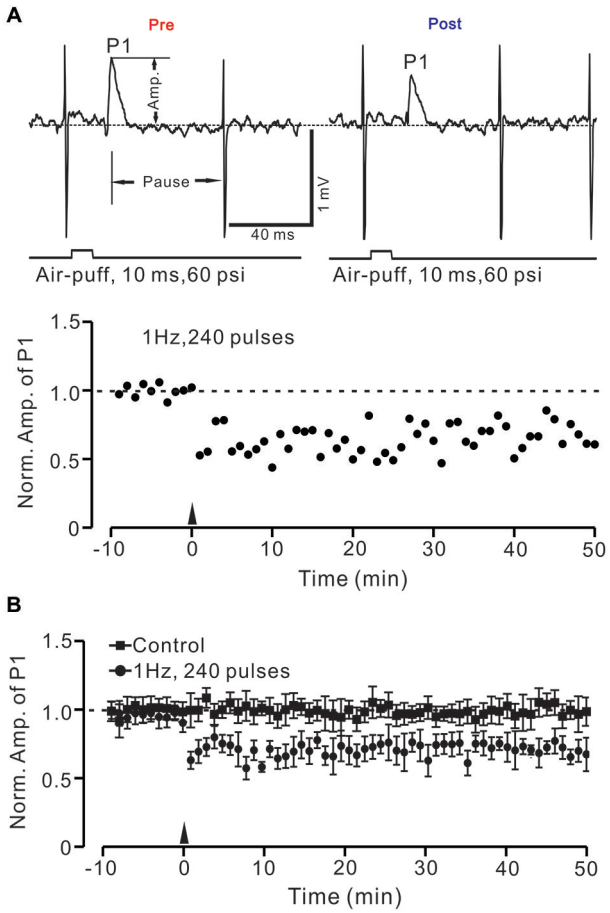

FIGURE 2 | Facial stimulation (1 Hz) induces long-term depression (LTD) of GABAergic transmission at PCs in mouse cerebellar cortex. **(A)** Upper, representative cell-attached recording traces showing air-puff stimulation (10 ms, 60 psi)-evoked responses in a cerebellar PC before (pre) and after (post) delivering 1 Hz (240 pulses) stimulation. Note that both amplitude of the positive component (P1) and the pause of SS firing are significantly decreased after 1 Hz stimulation. The lower panel shows the time course of normalized amplitude of P1 (shown in upper) before and after delivery of 1 Hz facial stimulation (arrow head). **(B)** Summary of normalized P1 amplitude under control conditions (squares; $n = 7$) and delivery of 1 Hz facial stimulation (arrow head; circles; $n = 8$). Note that air-puff stimulation at 1 Hz induced LTD of P1 in cerebellar PCs. Data points are mean ± SEM.

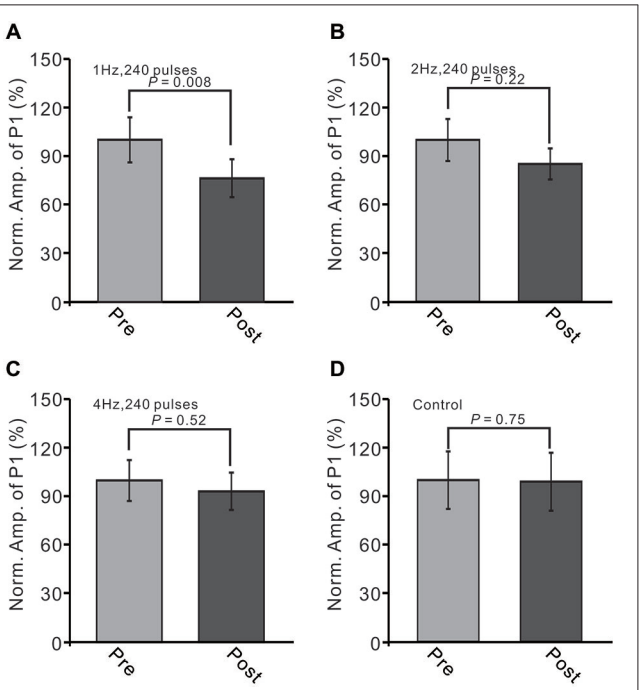

FIGURE 3 | LTD of sensory-evoked P1 at PCs was induced by 1 Hz, but not by 2 Hz or 4 Hz. **(A)** Bar graph showing normalized amplitude of P1 before (pre) and after (post) delivery of 2 Hz (240 pulses) stimulation. **(B)** Summary of data showing normalized amplitude of P1 before (pre) and after (post) 2 Hz (240 pulses) stimulation. **(C)** Pooled data showing normalized amplitude of P1 before (pre) and after (post) 4 Hz (240 pulses) stimulation. **(D)** Bar graph showing normalized amplitude of P1 under control conditions (without train of facial stimulation). Note that the amplitude of P1 was depressed after 1 Hz stimulation, but not after 2 or 4 Hz stimulation.

to 71.5 ± 8.9% of baseline (100.0 ± 11.6%; $P < 0.05$, $n = 7$; **Figure 3A**). However, after 2 Hz stimulation ($P > 0.05$, $n = 7$ in each group, **Figure 3B**), the normalized amplitude of P1 at 40–50 min was 84.8 ± 9.5% of baseline (99.6 ± 12.9%). After 4 Hz facial stimulation ($P > 0.05$, $n = 7$ in each group, **Figure 3C**), the normalized amplitude of P1 at 40–50 min was 93.3 ± 11.6% of baseline (100.0 ± 12.7%), which was similar to that under control conditions (99.9 ± 18.6% of baseline; $P > 0.05$, $n = 7$).

Additionally, we evaluated the change in the stimulation-evoked pause of spike firing after the trains of facial stimulation. The normalized value of the pause of spike firing at 40–50 min after 1 Hz facial stimulation was decreased to 76.3 ± 11.7% of baseline (100.0 ± 13.9%; $P < 0.05$, $n = 7$; **Figure 4A**). As with the normalized amplitude of P1, the normalized pause of spike firing at 40–50 min after 2 Hz facial stimulation was 91.4 ± 13.0% of baseline (99.8 ± 13.4%; $P > 0.05$, $n = 7$ in each group, **Figure 4B**), and the normalized pause of spike firing over 40–50 min after 4 Hz facial stimulation was 97.2 ± 8.3% of baseline (100.0 ± 9.6%; $P > 0.05$, $n = 7$ in each group, **Figure 4C**), which was similar to the normalized pause of spike firing over 40–50 min under control conditions (99.9 ± 18.6% of baseline; $P > 0.05$, $n = 7$; **Figure 4D**). These results indicated that LTD of GABAergic transmission at MLI–PC synapses could be induced by sensory stimulation at 1 Hz, but not at 2 Hz or 4 Hz.

Facial Stimulation (1 Hz) Induced LTD of GABAergic Transmission at PCs, but Without Significant Change in the Properties of Sensory-Evoked Spike Events in MLIs

MLIs receive sensory information from the MF–GC–PF pathway, and LTD of MLI–PC GABAergic transmission can be induced by the change of sensory stimulation-evoked MLI responses. Therefore, we examined the effects of 1 Hz facial stimulation on the activities of cerebellar MLIs. As with our previous study (Chu et al., 2012), cerebellar MLIs were identified by irregular spike firing and depth of recording sites

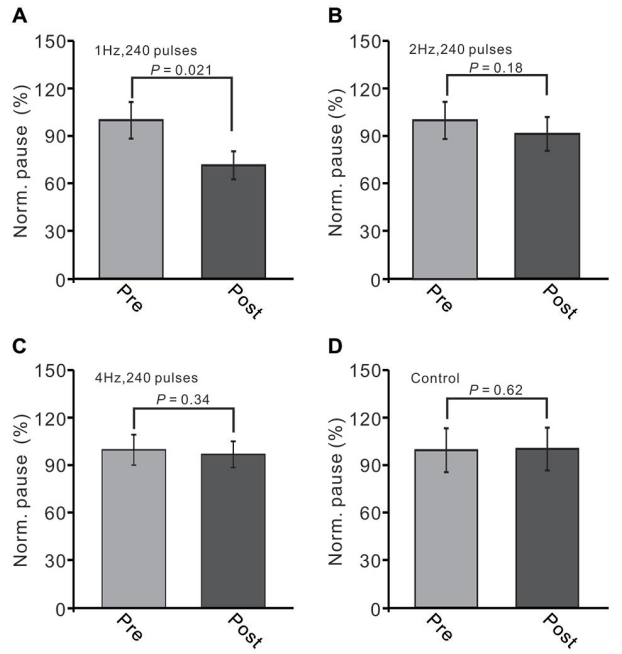

FIGURE 4 | LTD of sensory-evoked P1 at PCs was accompanied with a decrease in the pause of SS firing. (A) Bar graph showing normalized pause of SS firing before (pre) and after (post) delivery of 1 Hz (240 pulses) stimulation. **(B)** Pooled data showing normalized pause of SS firing before (pre) and after (post) delivery of 2 Hz (240 pulses) stimulation. **(C)** Summary of data showing normalized values of the pause before (pre) and after (post) delivering 4 Hz (240 pulses) stimulation. **(D)** Bar graph showing normalized pause firing values under control conditions (without train of facial stimulation). Note that the amplitude of the pause of SS firing was decreased after 1 Hz stimulation, but not after 2 Hz or 4 Hz stimulation.

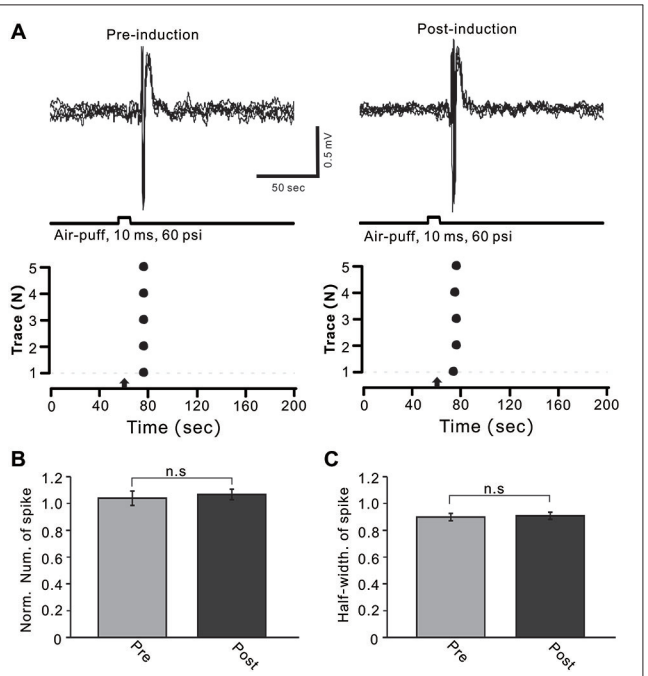

FIGURE 5 | Facial stimulation had a lesser effect on cerebellar MLI activity. **(A)** Upper, superposition of five representative recording epochs showing air-puff stimulation (10 ms, 60 psi)-evoked spike firing in an MLI before (left) and after (post) delivery of 1 Hz (240 pulses) stimulation. The lower panel shows the master plot of spike events derived from traces of the upper panel. **(B)** Summary of normalized number of spike events before (Pre, red) and after delivery of 1 Hz stimulation (Post; $n = 6$). **(C)** Pooled data showing the normalized half-width of the sensory-evoked spike events before (Pre) and after delivering 1 Hz stimulation (Post; $n = 6$). Note that air-puff stimulation at 1 Hz did not induce a significant change in the properties of the sensory-evoked spike events on MLIs.

(**Figure 1B**). Under cell-attached recording conditions, cerebellar MLIs exhibited spike firing in response to air-puff stimulation of the ipsilateral whisker pad (10 ms; 60 m psi; **Figure 5A**). After 1 Hz facial stimulation, the normalized number of evoked spike events at 40–50 min was 105.7 ± 5.3% of baseline (100.9 ± 3.9%), which was not significantly different from baseline ($P > 0.05$, $n = 7$ in each group, **Figures 5A,B**). Moreover, the mean half-width of the evoked spike events at 40–50 min was 0.90 ± 0.027 ms after 1 Hz facial stimulation, which was not significantly different from baseline (0.91 ± 0.026 ms; $P > 0.05$, $n = 7$ in each group, **Figures 5A,C**). These results indicated that air-puff stimulation at 1 Hz did not induce significant change in the properties of the sensory-evoked spike events on MLIs, suggesting that LTD of GABAergic transmission was induced by the train of sensory stimulation at MLI–PC synapses.

Induction of MLI–PC GABAergic LTD Requires Activation of CB1 Receptors

In the cerebellar cortex, eCBs are generated and released from PCs and MLIs by trains of PF stimulation via activation of mGluR1 and NMDA receptors (Beierlein and Regehr, 2006; Soler-Llavina and Sabatini, 2006), and are considered to be related to PF–PC presynaptic plasticity (Qiu and Knöpfel, 2009; Chu et al., 2014). Therefore, we examined the effect of the CB1 receptor antagonist on the induction of 1 Hz facial stimulation-induced LTD of GABAergic transmission at PCs. Blockade of endocannabinoid CB1 receptors with AM251 completely prevented the induction of 1 Hz facial stimulation-induced LTD of GABAergic transmission at MLI–PC synapses (**Figures 6A,B**). In the presence of AM251, the mean amplitude of P1 40–50 min after the trains of 1 Hz facial stimulation were delivered was 95.9 ± 9.4% ($n = 6$) of baseline (100 ± 5.2%; **Figures 6A,C**; $P < 0.05$), indicating that 1 Hz facial stimulation induced LTD of GABAergic transmission at MLI–PC synapses via CB1 receptor activation. Furthermore, we applied the CB1 receptor agonist, WIN55212-2, to examine whether LTD of GABAergic transmission at MLI–PC synapses could be induced by direct activation of CB1 receptors. As shown in **Figure 7**, application of WIN55212-2 (5 μM) for 10 min, induced a long-term depression in P1 amplitude (**Figures 7A,B**); the normalized P1 amplitude was 76.0 ± 5.9% of baseline (100 ± 4.5%) at 40–50 min ($P < 0.05$, $n = 7$; **Figure 7C**). These results indicated that induction of MLI–PC GABAergic LTD required CB1 receptor activation, which could be achieved by pharmacological activation.

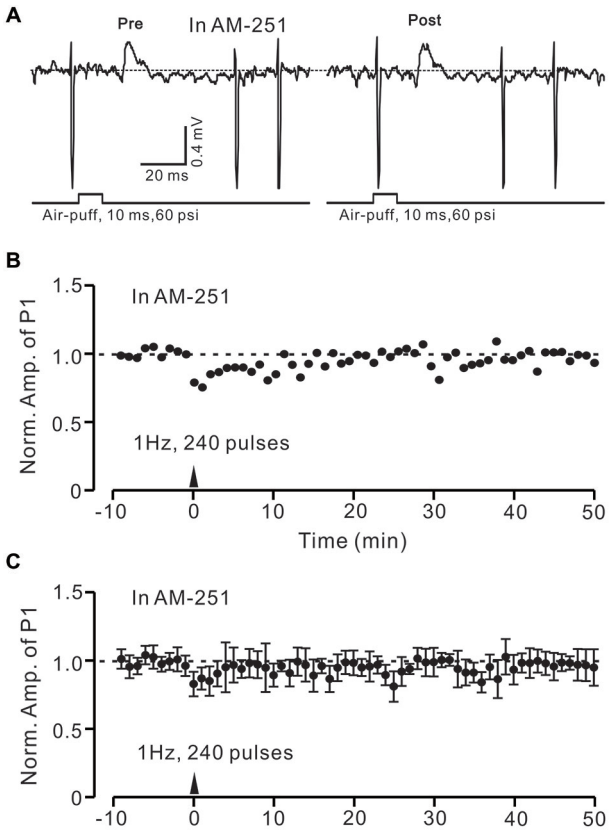

FIGURE 6 | Facial stimulation-induced LTD of GABAergic transmission at PCs was abolished by blocking cannabinoid type 1 (CB1) receptor activity. **(A)** Representative cell-attached recording traces showing air-puff stimulation (10 ms, 60 psi)-evoked responses in a cerebellar PC before (pre) and after (post) delivering 1 Hz (240 pulses) stimulation delivered in the presence of CB1 antagonist, AM251 (5 μM). **(B)** Time course of normalized amplitude of P1 (shown in **A**) before and after delivery of 1 Hz facial stimulation (arrow head) delivered in the presence of AM251. **(C)** Summary of normalized amplitude of P1 before and after delivery of 1 Hz (240 pulses) stimulation (arrow head; $n = 7$) delivered in the presence of AM251. Note that air-puff stimulation (1 Hz)-induced LTD of P1 was blocked by application of a CB1 receptor antagonist. Data points are mean ± SEM.

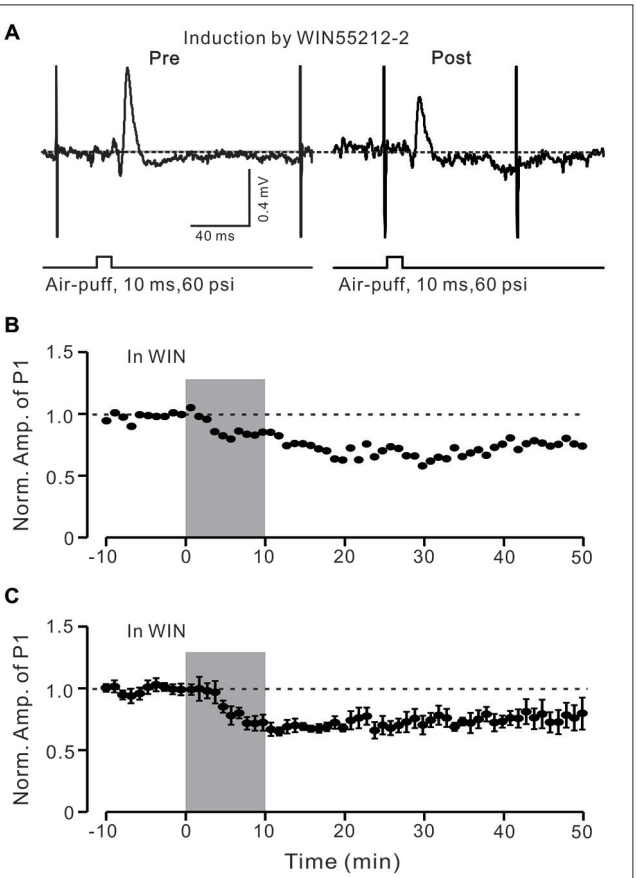

FIGURE 7 | LTD of GABAergic transmission at PCs was induced by a CB1 receptor agonist. **(A)** Representative cell-attached recording traces showing air-puff stimulation (10 ms, 60 psi)-evoked responses in a cerebellar PC before (left) and after application of CB1 agonist WIN55212-2 (5 μM; 10 min). **(B)** Time course of normalized amplitude of P1 (shown in **A**) before and after application of CB1 agonist WIN55212-2 (5 μM; 10 min; shadow). **(C)** Pooled data showing the average normalized amplitude of P1 before and after application of CB1 agonist WIN55212-2 (5 μM; 10 min; shadow; $n = 7$). Note that application of CB1 agonist WIN55212-2 for 10 min induced LTD of P1. Data points are mean ± SEM.

Induction of MLI–PC GABAergic LTD Requires Activation of NMDA Receptors, but not mGluR1

In the cerebellar cortex, PCs and MLIs generate and release eCB by mGluR1 activation (Brown et al., 2003; Beierlein and Regehr, 2006; Soler-Llavina and Sabatini, 2006). Therefore, we examined whether MLI–PC synaptic transmission LTD could be induced by 1 Hz stimulation delivered with blocking of mGluR1. When mGluR1 activity was blocked during 1 Hz stimulation, a persistent depression of MLI–PC GABAergic synaptic transmission was still induced (**Figure 8A**). The normalized amplitude of P1 was decreased to 78.3 ± 7.65% of baseline (100.0 ± 5.9%) at 40–50 min after 1 Hz facial stimulation ($P < 0.05$; $n = 6$; **Figure 8B**). The normalized value of the pause of spike firing at 40–50 min after 1 Hz facial stimulation was decreased to 81.2 ± 5.1% of baseline (102.2 ± 6.1%; $P < 0.05$, $n = 6$; **Figure 8C**). These results indicate that mGluR1 activity contributed less to the induction of MLI–PC GABAergic LTD under *in vivo* conditions.

NMDA receptor activation is required for eCB-dependent presynaptic LTD induction, both in mouse cerebellar slices (Qiu and Knöpfel, 2009) and in living animals (Chu et al., 2014). Thus, we further examined whether the LTD of MLI–PC synaptic transmission could be induced by 1 Hz stimulation delivered without the activity of NMDA receptors. When NMDA receptor activity was blocked during 1 Hz stimulation with D-APV (50 μM), the LTD of MLI–PC GABAergic synaptic transmission was not induced by facial stimulation (**Figure 9A**). The normalized amplitude of P1 was 101.3 ± 12.9% of baseline (100.0 ± 6.3%) at 40–50 min after 1 Hz facial stimulation ($P > 0.05$, $n = 6$; **Figure 9B**). The normalized value of the pause of spike firing at 40–50 min was 102.0 ± 9.3% of baseline (100.0 ± 8.6%) after 1 Hz facial stimulation ($P > 0.05$, $n = 6$; **Figure 9C**).

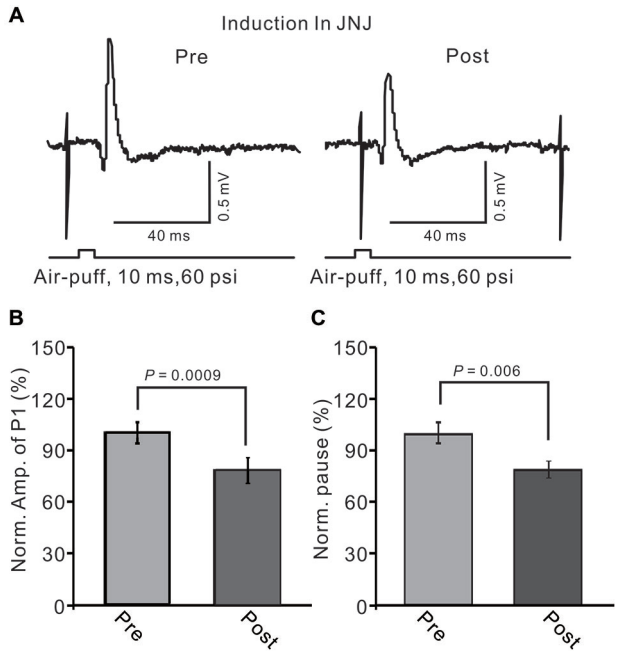

FIGURE 8 | Blocking metabotropic glutamate receptor 1 (mGluR1) during 1 Hz stimulation failed to prevent the LTD of GABAergic transmission at PCs. **(A)** Upper, representative cell-attached recording traces showing air-puff stimulation (10 ms, 60 psi)-evoked responses in a PC before (pre) and after (post) 1 Hz (240 pulses) stimulation delivered in the presence of mGluR1 antagonist, JNJ16259685 (10 μM, right panel). **(B)** Summary of data showing normalized amplitude of P1 before (pre) and after (post) delivery of 1 Hz (240 pulses) stimulation. **(C)** Pooled data showing the normalized pause of SS firing before (pre) and after (post) delivery of 1 Hz (240 pulses) stimulation. Note that the amplitude of P1 was still depressed after 1 Hz stimulation without mGluR1 activity.

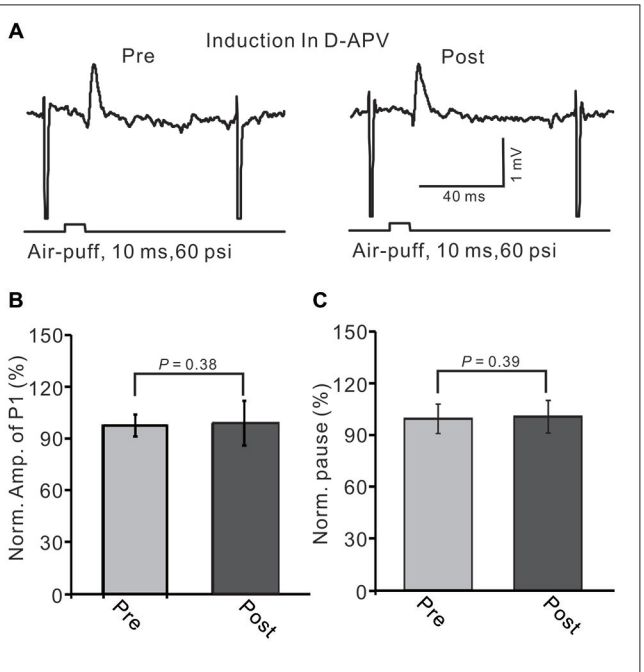

FIGURE 9 | Blocking N-methyl-D-aspartate (NMDA) receptors during 1 Hz stimulation abolished the LTD of GABAergic transmission at PCs. **(A)** Upper, representative cell-attached recording traces showing air-puff stimulation (10 ms, 60 psi)-evoked responses in a PC before (pre) and after (post) delivery of 1 Hz (240 pulses) stimulation delivered in the presence of NMDA receptor antagonist, D-APV (50 μM). **(B)** Summary of data showing normalized amplitude of P1 before (pre) and after (post) delivery of 1 Hz (240 pulses) stimulation. **(C)** Pooled data showing the normalized pause of SS firing before (pre) and after (post) delivery of 1 Hz (240 pulses) stimulation.

These results indicate that the induction of MLI–PC GABAergic LTD under *in vivo* conditions occurs via the activation of NMDA receptors.

Discussion

Our major finding is that 1 Hz, but not 2 Hz or 4 Hz, facial stimulation induces GABAergic transmission LTD at MLI–PC synapses. The MLI–PC GABAergic LTD could be prevented by blocking CB1 receptors, and could be mimicked by pharmacological activation of CB1 receptors. Additionally, the MLI–PC GABAergic LTD was abolished by blocking NMDA receptor activity during inductive stimulation. Our present results indicate that sensory stimulation induces an eCB-dependent LTD of GABAergic transmission at MLI–PC synapses via NMDA receptor activation in living animals.

Repeated Facial Stimulation Induces LTD of GABAergic Transmission at MLI–PC Synapses *In Vivo* in Mice

MLI–PC synaptic plasticity induced by postsynaptic depolarization at SC–PC synapses under *in vitro* conditions involves presynaptic CB1 receptors (Llano et al., 1991a; Yoshida et al., 2002), NMDA receptors (Duguid and Smart, 2004), and postsynaptic $GABA_A$ receptors (Hirano and Kawaguchi, 2014). In the present study, MLI–PC GABAergic transmission LTD was induced by 1 Hz, but not 2 Hz or 4 Hz, sensory stimulation *in vivo* in mice, suggesting that sensory stimulation-evoked LTD of MLI–PC synaptic transmission is stimulation-frequency-dependent. The cerebellar MLI network acts as a low-pass filter during the processing of high-frequency sensory information, and plays a critical role in the sensory-related outputs of PCs in the cerebellar cortex (Chu et al., 2012; Bing et al., 2015a). However, cerebellar PCs generate sensory-related output that is limited to low-frequency sensory stimulation, and is independent of $GABA_A$ receptor-mediated inhibition (Bing et al., 2015b). Therefore, MLI–PC GABAergic transmission LTD was induced by 1 Hz, but not 2 Hz or 4 Hz, sensory stimulation.

In terms of cerebellar cortical circuitry, MLIs receive sensory information from the MF–GC–PF pathway, which evokes strong GABAergic inhibition rather than excitation in cerebellar PCs (Chu et al., 2011a,b). Therefore, LTD of MLI–PC GABAergic transmission was induced by depression of the sensory stimulation-evoked spike firing of MLIs, such as the sensory stimulation-evoked LTD of MF–GC synapses and/or PF–MLI synapses. Indeed, it is reported that sensory stimulation at 4 Hz can induce LTD at MF–GC synapses in anesthetized rats (Roggeri et al., 2008). PF–PC LTD could occur together with PF–MLI LTP and MLI–PC

inhibitory LTP, while PF–PC LTP could occur together with PF–MLI LTD and MLI–PC inhibitory LTD (Gao et al., 2012). However, our results show that air-puff stimulation at 1 Hz does not change the properties of sensory-evoked spike events on MLIs, suggesting that GABAergic transmission LTD is induced by repeated sensory stimulation at MLI–PC synapses.

Possible Mechanisms of Sensory-Induced MLI–PC GABAergic LTD *In Vivo* in Mice

Our present results show that MLI–PC GABAergic LTD can be prevented by blocking CB1 receptor activity, and can be pharmacologically induced by a CB1 receptor agonist, indicating that sensory stimulation induces GABAergic transmission LTD at MLI–PC synapses via the eCB signaling pathway. eCB-mediated LTD, has been reported to be induced in several brain areas at both inhibitory and excitatory synapses (Chevaleyre et al., 2006). In the hippocampus, stimulation of Schaffer collaterals induces CB1 receptor-dependent LTD at presynaptic GABAergic terminals via activation of mGluR1 (Chevaleyre et al., 2007). In the cerebellar cortex, eCB release from other PCs can be triggered by activation of mGluR1 following the spontaneous CF inputs (Safo et al., 2006). In the cerebellar cortex, CB1 receptor-dependent PF–PC presynaptic LTD was observed *in vivo* in the absence of a pharmacological blocker, suggesting that eCB signaling under *in vivo* conditions is stronger than that under *in vitro*. A presynaptically expressed form of eCB-dependent LTD has been suggested at PF–MLI synapses (Soler-Llavina and Sabatini, 2006). Therefore, eCB signaling plays a critical role in cerebellar cortical neuronal plasticity.

Additionally, mGluR1 activation could induce eCB generation and release from cerebellar PCs and MLIs (Brown et al., 2003; Beierlein and Regehr, 2006; Soler-Llavina and Sabatini, 2006). However, our present results show that MLI–PC GABAergic transmission LTD can be induced in the presence of an mGluR1 blocker, indicating that mGluR1 plays a non-essential role during facial stimulation-evoked LTD of MLI–PC GABAergic transmission of PCs. In fact, facial stimulation evokes inhibition rather than excitation in cerebellar PCs under control conditions (Chu et al., 2011a,b); thus, the inhibition of PCs during sensory stimulation resulted in a lower amount of eCB released by activation of mGluR1. Interestingly, blockade of NMDA receptors during 1 Hz facial stimulation abolished the expression of MLI–PC GABAergic LTD, indicating that the sensory stimulation induced eCB-dependent LTD of GABAergic transmission at MLI–PC synapses via activation of NMDA receptors. Although electrophysiological recordings have shown that functional NMDA receptors are no longer expressed in cerebellar PCs after the first postnatal week (Konnerth et al., 1990; Llano et al., 1991b), postsynaptic functional NMDA receptors are prominent in CF–PC synapses in adult mice (Piochon et al., 2007; Renzi et al., 2007; Bidoret et al., 2009). Importantly, NMDA receptor subunits have been found in cultured MLIs (Duguid and Smart, 2004) and on the axonal pinceau of basket-type MLIs (Petralia et al., 1994). The presence of NMDA receptors on MLIs is the likely a source of eCB (Beierlein and Regehr, 2006) for presynaptic LTD (Qiu and Knöpfel, 2009; Li and Burrell, 2011). Indeed, NMDA receptor activation is required for eCB-dependent presynaptic LTD induction when presynaptic LTP is pharmacologically blocked both in mouse cerebellar slices (Qiu and Knöpfel, 2009) and *in vivo* in mice (Chu et al., 2014). The present results show that the expression of MLI–PC GABAergic LTD is dependent on the activity of NMDA receptors, but that 1 Hz sensory stimulation did not induce a significant change in the properties of sensory-evoked spike events on MLIs, suggesting that LTD of GABAergic transmission might involve presynaptic NMDA receptors. Presynaptic NMDA receptors are thought to act as local high-gain glutamate detectors in cerebellar MLIs (Rossi and Collin, 2013), and the activation of presynaptic NMDA receptors in MLIs can induce an increase in GABA release into MLI–PC terminals (Bouhours et al., 2011) Additionally, activated NMDA receptors trigger the activation of nitric oxide (NO) synthesis and the release of NO from MLIs (Akazawa et al., 1994; Carter and Regehr, 2000). NO works as retrograde signal, and has been implicated in various forms of presynaptically expressed LTP (Hardingham and Fox, 2006; Qiu and Knöpfel, 2007; Chu et al., 2014). However, it seems that NO contributes less to the LTD of GABAergic transmission at MLI–PC synapses under *in vivo* conditions (data not shown).

Taken together, our present study demonstrates that 1 Hz, but not 2 Hz or 4 Hz, facial stimulation induces an eCB-mediated LTD of GABAergic transmission at MLI–PC synapses via activation of NMDA receptors in cerebellar cortical Crus II *in vivo* in mice. Our results highlight that eCB retrograde signaling is activated by sensory stimulation and is necessary for the induction of LTD of MLI–PC GABAergic transmission in living animals.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (31060138; 81260208; 81160142; 31460261; 20150101070JC).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in
CELLULAR NEUROSCIENCE

ORIGINAL RESEARCH ARTICLE
published: 02 February 2015
doi: 10.3389/fncel.2015.00010

The role of cAMP in synaptic homeostasis in response to environmental temperature challenges and hyperexcitability mutations

Atsushi Ueda and Chun-Fang Wu*

Department of Biology, University of Iowa, Iowa City, IA, USA

Edited by:
Jaichandar Subramanian, Massachusetts Institute of Technology, USA

Reviewed by:
Subhabrata Sanyal, Emory University, USA
Mani Ramaswami, Trinity College Dublin, Afghanistan

***Correspondence:**
Chun-Fang Wu, Department of Biology, University of Iowa, 129 E. Jefferson St., Biology Building, Room 231, Iowa City, IA 52242, USA
e-mail: chun-fang-wu@uiowa.edu

Homeostasis is the ability of physiological systems to regain functional balance following environment or experimental insults and synaptic homeostasis has been demonstrated in various species following genetic or pharmacological disruptions. Among environmental challenges, homeostatic responses to temperature extremes are critical to animal survival under natural conditions. We previously reported that axon terminal arborization in *Drosophila* larval neuromuscular junctions (NMJs) is enhanced at elevated temperatures; however, the amplitude of excitatory junctional potentials (EJPs) remains unaltered despite the increase in synaptic bouton numbers. Here we determine the cellular basis of this homeostatic adjustment in larvae reared at high temperature (HT, 29°C). We found that synaptic current focally recorded from individual synaptic boutons was unaffected by rearing temperature (<15°C to >30°C). However, HT rearing decreased the quantal size (amplitude of spontaneous miniature EJPs, or mEJPs), which compensates for the increased number of synaptic releasing sites to retain a normal EJP size. The quantal size decrease is accounted for by a decrease in input resistance of the postsynaptic muscle fiber, indicating an increase in membrane area that matches the synaptic growth at HT. Interestingly, a mutation in *rutabaga* (*rut*) encoding adenylyl cyclase (AC) exhibited no obvious changes in quantal size or input resistance of postsynaptic muscle cells after HT rearing, suggesting an important role for *rut* AC in temperature-induced synaptic homeostasis in *Drosophila*. This extends our previous finding of *rut*-dependent synaptic homeostasis in hyperexcitable mutants, e.g., *slowpoke* (*slo*). In *slo* larvae, the lack of BK channel function is partially ameliorated by upregulation of presynaptic Shaker (Sh) IA current to limit excessive transmitter release in addition to postsynaptic glutamate receptor recomposition that reduces the quantal size.

Keywords: rutabaga adenylyl cyclase, quantal size, input resistance, synaptic growth, quantal content

INTRODUCTION

Homeostatic mechanisms are involved in the striking ability of regaining stable synaptic efficacy or neural circuit performance following disturbances caused by environmental stressors or experimental insults in the nervous systems of a variety of species (Turrigiano and Nelson, 2004; Pérez-Otaño and Ehlers, 2005; Davis, 2006; Marder and Goaillard, 2006). In mammals, pharmacological manipulations of neuronal spiking or synaptic activities have been shown to cause compensatory changes in synaptic strength. For example, the blockade of action potentials by Tetrodotoxin (TTX) or postsynaptic inhibition by receptor antagonists can result in striking increases in spontaneous excitatory synaptic currents (EPSCs), as observed in dissociated cultures of cortical neurons (Turrigiano et al., 1998), hippocampal slices (Rao and Craig, 1997; Lissin et al., 1998), and *in vivo* recordings of visual cortical neurons (Desai et al., 2002) and spinal neurons (O'Brien et al., 1998). In some cases, the compensatory response is mediated through upregulation of receptor subtype expression (Watt et al., 2000; Leslie et al., 2001). However, the underlying general regulatory mechanisms and detailed molecular networks still await further elucidation.

A naturally occurring and ecologically relevant stressor that induces neuronal homeostatic adjustment is environmental temperature. Chronic temperature changes are known to affect neuronal development, leading to neuronal morphological alterations, such as neuronal dendritic field retraction during hibernation in ground squirrels (Popov and Bocharova, 1992; Popov et al., 1992; von der Ohe et al., 2006) or dendritic spine number reduction following short-term decreases in local temperature in the mouse brain (Kirov et al., 2004; Roelandse and Matus, 2004). Synaptic homeostatic adjustments also maintain the stability of physiological functions upon drastic temperature changes. For instance, in the stomatogastric ganglion of the crab phase relationships between action potential bursts characteristic to individual identified neurons are maintained

despite temperature changes that significantly alter the neuronal firing rate within the central pattern generators (Tang et al., 2010, 2012). It is also known that after long-term exposure to extreme temperatures, neurons display adjustments in various physiological parameters, such as resting membrane potential and EPSP amplitude, in a number of species (fish: Roots and Prosser, 1962; Friedlander et al., 1976; snail: Merickel and Kater, 1974; crayfish: Harri and Florey, 1979; honeybee: Tautz et al., 2003; Groh et al., 2004; Jones et al., 2005).

The *Drosophila* larval neuromuscular junction (NMJ) offers abundant opportunities for studying the molecular and genetic mechanisms underlying synaptic homeostasis (Davis and Bezprozvanny, 2001 for review). It has been documented in *Drosophila* that axon terminal arborization is enhanced at increased temperatures (Sigrist et al., 2003; Zhong and Wu, 2004; Peng et al., 2007; Lee and Wu, 2010). These temperature-induced morphological alterations can be further modulated by mutations altering neuronal excitability: drastic increases by K^+ channel mutations (Budnik et al., 1990; Zhong et al., 1992) and decreases by Ca^{2+} channel mutations (Lee and Wu, 2010). Significantly, such morphological modifications induced by increased temperature or neuronal hyperexcitability can be suppressed by a mutation in *rutabaga* (*rut*) encoding adenylyl cyclase (AC), implicating the involvement of the cAMP pathway in the plasticity of synaptic growth (Zhong et al., 1992; Lee and Wu, 2010).

Here we examine the physiological parameters of pre- and post-synaptic elements to identify the temperature-dependent alterations in maintaining. Such counterbalancing modifications enable the maintenance of stable synaptic transmission at the *Drosophila* larval NMJ upon high temperature (HT) rearing. In spite of the increased number of synaptic boutons, the excitatory junctional potential (EJP) size remains unaltered. Strikingly, this temperature-induced synaptic homeostasis was not observed in mutant larvae with impaired function of *rut* AC following HT rearing.

MATERIALS AND METHODS

DROSOPHILA STOCKS

The *Drosophila melanogaster* stocks used include a wild-type (WT) strain Canton-S and a mutant *rutabaga*^{1} (*rut*^{1}). These lines have been previously described (Zhong et al., 1992; Kim and Wu, 1996; Renger et al., 2000; Peng et al., 2007). Flies carrying *UAS-rut*$^+$ was a generous gift from Dr. Troy Zars (University of Missouri, Columbia, MO, USA; Zars et al., 2000). A motor neuron-specific driver *C164-Gal4* (Torroja et al., 1999) and muscle-specific driver *mef2-Gal4* (Ranganayakulu et al., 1996) were used to drive expression of the transgene.

Fly stocks were maintained at room temperature (RT). However, the building RT varied significantly over the seasons between 1997 and 2001, as low as 15°C during winter and as high as 30°C during summer. Focal recording was carried out during this period. Experiments on rearing temperature effects and intracellular recording of EJP were performed after 2002 when the building temperature was maintained at 22–24°C throughout the year. To examine the effect of rearing temperature, we compared the stocks maintained at RT with those reared in 29–30°C incubators.

LARVAL NEUROMUSCULAR PREPARATIONS AND PHYSIOLOGICAL SOLUTIONS

Post-feeding third instar larvae were dissected in Ca^{2+} free HL3 saline (Stewart et al., 1994) containing (in mM) 70 NaCl, 5 KCl, 20 $MgCl_2$, 10 $NaHCO_3$, 5 Trehalose, 115 Sucrose, and 5 HEPES, at pH 7.2. For physiological recordings, we used either HL3 (focal recording in **Figures 2**, **4**) or HL3.1 (Feng et al., 2004), which have the same ionic composition except for a reduced Mg^{2+} concentration in HL3.1 (4 mM, whole cell intracellular recordings, **Figures 1**, **3**, **5**, **6**). The final Ca^{2+} concentration in recording saline is specified for each experiment. To evoke nerve action potentials and excitatory junctional currents (EJCs), the segmental nerves were severed from the ventral ganglion and stimulated with a suction electrode (10 μm inner diameter) through the cut end. Stimulation amplitude was adjusted to 2.0–2.5 times the threshold voltage to ensure a uniform stimulation condition among experiments. Stimulus duration was 0.1 or 0.5 ms.

FOCAL LOOSE PATCH-CLAMP RECORDING

Extracellular focal recordings were performed as described previously (Renger et al., 2000; Ueda and Wu, 2009). Briefly, fire-polished focal recording electrodes with an inner diameter of 4–8 μm and an outer diameter of 15–20 μm were filled with HL3 saline and were placed over type I boutons on muscle 13. The pipette opening typically covered one type Ib bouton. EJC signals were picked up with a loose-patch clamp amplifier (Patch Clamp 8510; Zeitz Instruments, Munich, Germany) and stored on VCR tapes with a Pulse Code Modulator (Neuro Data, model Neuro-Corder DR-384, New York, NY). All trials contained a calibration pulse to determine the electrode series and seal resistance in order to correct for current leakage at the pipette tip (Stühmer et al., 1983). In rare occasions, biphasic currents were observed, but they were excluded from data analysis.

WHOLE-CELL EXCITATORY JUNCTIONAL POTENTIAL (EJP) AND MUSCLE INPUT RESISTANCE MEASUREMENTS

Nerve-evoked neurotransmitter release was also recorded intracellularly from postsynaptic muscle fiber 6. Intracellular glass microelectrodes were filled with 3 M KCl and had a series resistance of about 60 MΩ. EJPs were picked up with a direct current pre-amplifier (model M701 micro-probe system, WPI, Conn., USA, and an additional custom-built amplifier). Muscle membrane resistance was measured by injecting −1 nA current pulses with 700 ms duration into a muscle cell. A bridge circuit was used to measure the membrane potential change. We selected cells with resting potentials deeper than −55 mV for evoked EJPs and muscle input resistance measurement. For miniature EJP (mEJP) measurement, we selected cells with resting potentials deeper than −60 mV to ensure minimal membrane damage caused by electrode penetrations.

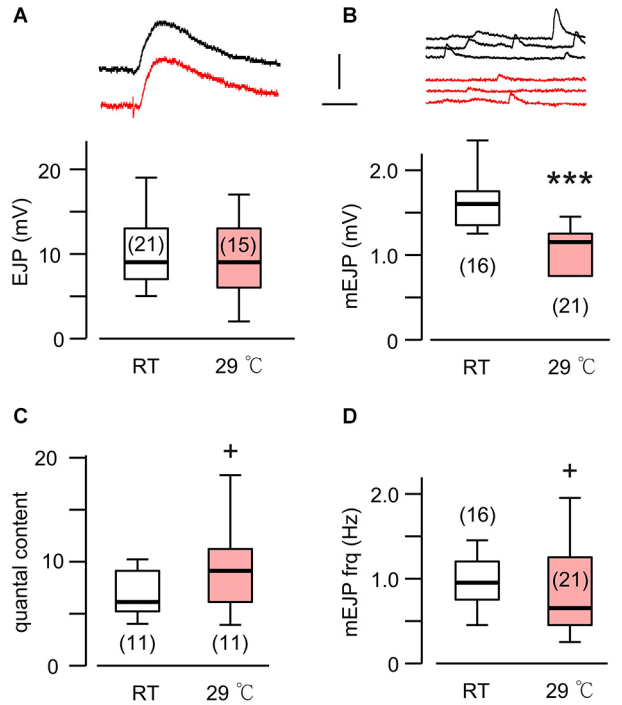

FIGURE 1 | A novel form of synaptic homeostasis in response to high temperature rearing. **(A)** Evoked EJP size was not affected by rearing temperatures. Upper (black) and lower (red) traces are from WT larvae reared at room temperature (23°C, RT) and high temperature (29°C, HT), respectively. **(B)** Miniature EJPs (mEJPs) became smaller when reared at HT. ***$p < 0.001$, *t*-test. **(C)** Quantal content (ratio of mean amplitudes of EJP to mEJP) in HT-reared larvae could be larger than that in RT-reared larvae, as indicated by a skewed distribution. + $p < 0.05$, *F*-test. **(D)** Frequency of mEJPs in HT-reared larvae was also more variable than that in RT-reared larvae. + $p < 0.05$, *F*-test. Scale bars: 5 mV and 20 ms **(A)** or 200 ms **(B)**. Number of muscle fibers indicated in parenthesis. Data recorded in HL3.1 saline containing 0.2 mM **(A,C)** or 0.2 and 0.5 mM Ca^{2+} **(B,D)**. **(A)** is constructed from data in Ueda and Wu (2012) with additional data.

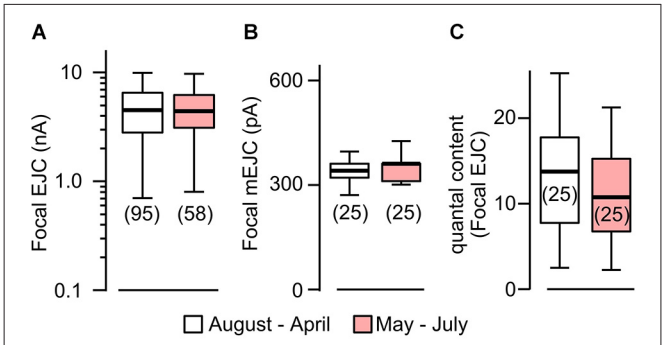

FIGURE 2 | Focal mEJCs and EJCs were not affected by rearing temperature. Focal EJCs **(A)** and mEJCs **(B)** remained stable despite ambient temperature fluctuations. Building temperature varied significantly: from as low as 15°C to beyond 30°C (mostly during May to July). Pooled data from August to April are compared to those from May to July. **(C)** Quantal content of the focal EJCs was not different between the two periods ($p > 0.5$, Wilcoxon rank-sum test). Number of recording sites indicated in parenthesis. **(A)** is reconstructed from data in Ueda and Wu (2012).

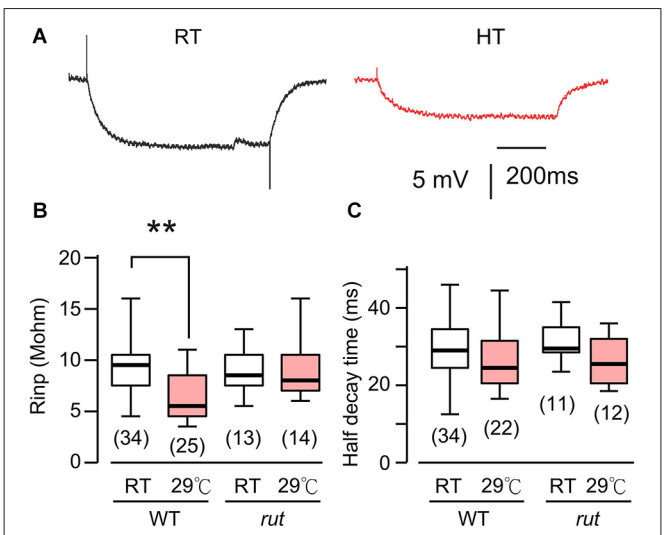

FIGURE 3 | HT rearing decreased muscle input resistance in WT, but not *rut* larvae. **(A)** Muscle membrane potential traces in response to hyperpolarizing current injection in WT larvae reared at RT and HT. **(B)** Input resistance was decreased in HT-reared WT, but not *rut*, larvae. **(C)** Rearing temperature did not significantly affect half-decay time in WT and *rut*. *$p <$ 0.05, One-way ANOVA. Number of muscle fibers indicated in parenthesis. Data from 0.2 and 0.5 mM Ca^{2+} were pooled. HL3.1 saline.

STATISTICAL ANALYSIS

As described in the Results, ANOVA, *F*-test, Wilcoxon rank-sum test, and student *t*-test were carried out with sequential Bonferroni adjustment for multiple comparisons.

RESULTS

HOMEOSTASIS OF SYNAPTIC EFFICACY UPON HT REARING: MAINTAINED EJP AMPLITUDE WITH INCREASED BOUTON NUMBERS AND REDUCED mEJP SIZE

It is now well established that HT rearing (at 29°C) induces synaptic terminal overgrowth resulting in an increased number of presynaptic boutons at the *Drosophila* larval NMJ (Sigrist et al., 2003; Zhong and Wu, 2004; Lee and Wu, 2010). However, the synaptic strength, as determined by the amplitude of the EJPs in the postsynaptic muscle cell, remains unaltered compared to that in larvae reared at RT (**Figure 1A**; Ueda and Wu, 2012). This raises the question of how the number of presynaptic vesicles released from individual boutons and the postsynaptic response to individual vesicles are adjusted in order to maintain stable synaptic efficacy when reared at HT. To answer this question, we first carried out intracellular recording to measure the muscle whole-cell response (EJP) and the spontaneous miniature EJPs (mEJPs), which reflect the postsynaptic response to spontaneous release of single vesicles (quantal size, **Figure 1B**). These parameters allowed us to estimate the quantal content, i.e., the number of vesicles released, underlying each EJP.

We used saline containing low Ca^{2+} (0.2 mM), at which the summation of mEJPs is nearly linear, circumventing the

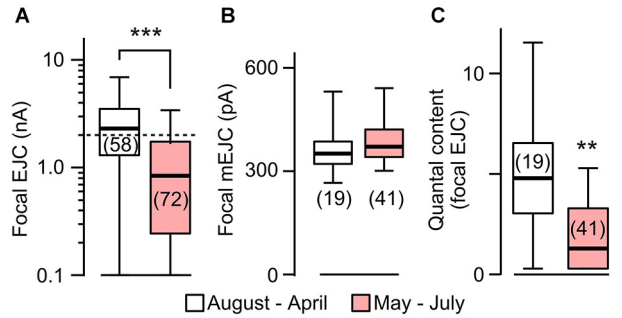

FIGURE 4 | Focal EJC amplitudes affected by rearing temperature in *rut*. **(A)** Focal EJCs from the great majority of *rut* boutons were below 2 nA (dotted line) during the period of May to July, whereas those collected outside of this period were substantially larger. Compare to **Figure 2** for constant EJC size for WT. **(B)** Focal mEJC amplitudes in *rut* did not vary with ambient temperature fluctuations. **(C)** Quantal content was significantly smaller at HT. Number of recording sites indicated in parenthesis. ***$p < 0.001$; **$p < 0.01$, Wilcoxon rank-sum test. **(A)** is reconstructed from data in Ueda and Wu (2012).

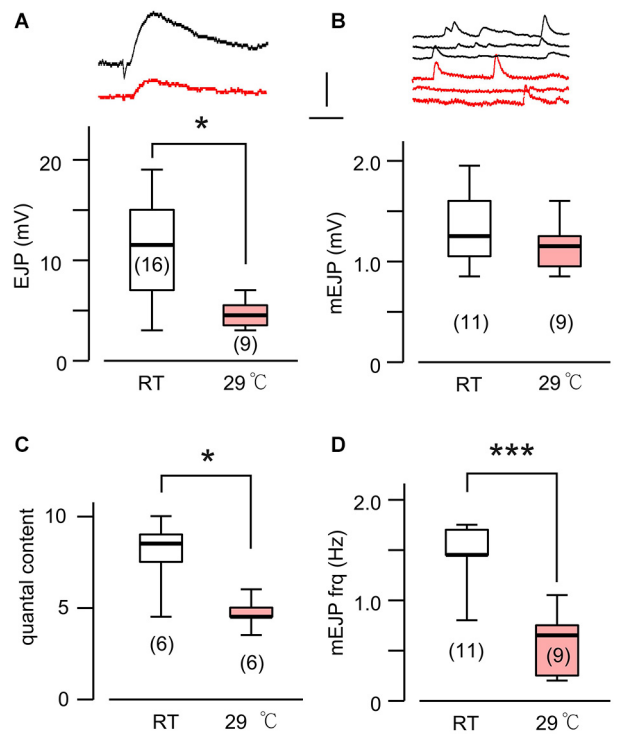

FIGURE 5 | Altered transmitter release in HT-reared *rut* larvae. **(A)** Evoked EJP size became drastically smaller in *rut* when reared at HT. Upper (black) and lower (red) traces are from RT- and HT- reared larvae, respectively. In *rut*, mEJP size was not significantly altered by HT rearing **(B)**, although quantal content was significantly reduced **(C)**. Frequency of mEJP was reduced in *rut* when reared at HT **(D)**. ***$p < 0.001$; *$p < 0.05$, *t*-test. Number of muscle fibers indicated in parenthesis. Data recorded in HL3.1 saline containing 0.2 mM **(A,C)** or 0.2 and 0.5 mM Ca^{2+} **(B,D)**. **(A)** is constructed from data in Ueda and Wu (2012) with additional data.

problem of nonlinear summation, allowing for the simple determination of quantal contents by the index [EJP]/[mEJP],

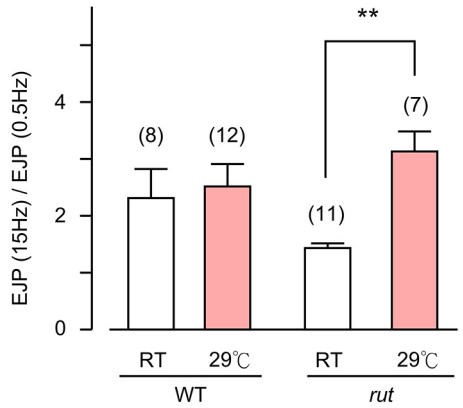

FIGURE 6 | Altered activity-dependent facilitation of EJPs in *rut* larvae. EJPs in *rut* larvae displayed enhanced facilitation when reared at HT. Ratios of EJP amplitudes evoked by 15-Hz–0.5 Hz provides an index for facilitation. Stimulus trains (10 s, 15 Hz) were applied and average EJP amplitudes were determined between 5–10 s. **$p < 0.01$, *t*-test with sequential Bonferroni adjustment for multiple comparisons of RT vs. HT. Number of muscle fibers indicated in parentheses. Error bars indicate SEM.

where [EJP] and [mEJP] represent the average amplitude of EJPs and mEJPs. Our data collected from muscles 6 and 7 indicate that the quantal size, as determined from the mEJP size, was reduced in WT when reared at HT (**Figure 1B**). To maintain the same EJP size, this led to an increase in quantal content (**Figure 1C**). Interestingly, a previous study also reported a similar percentage increase in the number of boutons in muscles 6 and 7 after HT rearing at 29°C (Sigrist et al., 2003). Therefore, it is important to investigate how HT-rearing affects the functioning at the synaptic bouton level, in terms of the number of vesicles released upon nerve stimulation and the postsynaptic currents generated by each vesicle in order to account for the ensemble EJP response evoked by each nerve action potential.

REARING-TEMPERATURE EFFECT ON POSTSYNAPTIC CURRENTS GENERATED BY INDIVIDUAL BOUTONS

We have previously established focal loose-patch recordings to determine local synaptic currents generated from transmitter release within individual boutons (Kurdyak et al., 1994; Renger et al., 2000; Ueda and Wu, 2009, 2012). Using a focal pipet electrode of a standard configuration and size (see Section Materials and Methods), local EJCs, as well as spontaneous miniature EJCs (mEJCs), from boutons under the patch electrode can be determined to complement whole-cell intracellular recordings of spontaneous mEJPs and evoked EJPs.

To quantify temperature-induced changes in focal mEJCs and EJCs, we performed loose-patch clamping followed by the established procedure to correct for errors introduced by leakage currents (see Section Materials and Methods; Kurdyak et al., 1994). We took advantage of a large data set collected between 1997–2000, during which ambient temperature for fly rearing and physiological recording varied between different seasons, approaching 30°C in the summers and 15°C in

the winters (see Section Materials and Methods; Ueda and Wu, 2012). We followed chronologically the data throughout these years and found no variation in EJC amplitude despite the temperature variation (**Figure 2A**; Ueda and Wu, 2012). When we performed additional analysis to include mEJC size using the same set of focal recording data, we found that focal mEJC size was also unaltered (**Figure 2B**). The fact that focal mEJCs remained the same is consistent with the simple interpretation that rearing temperature affects neither the amount of transmitter released per synaptic vesicle, nor the postsynaptic glutamate receptor channel response to each vesicle release. Therefore, unaltered focal EJCs and mEJCs support the conclusion that the quantal content for the local release from each bouton was unaffected by temperature variations (**Figure 2C**). Overall, this is consistent with the notion that the increased whole-cell quantal content determined by EJP measurements (**Figure 1C**) simply reflects the increase in presynaptic bouton numbers.

It should be noted that focal mEJCs amplitude did not appear to vary at different Ca^{2+} levels. Although the focal mEJC analyses in **Figure 2** were performed at a physiological Ca^{2+} level (1.5 mM), the results were compatible with the whole-cell mEJP recording that were collected at a low Ca^{2+} concentration (0.2 mM, **Figure 1** to avoid nonlinear summation distortion, see Section Materials and Methods). Additional focal mEJC measurements performed at a lower Ca^{2+} concentration (0.5 mM) produced similar results (330 ± 67 pA at 0.5 mM, $n = 10$ fibers vs. 340 ± 45 pA at 1.5 mM, $n = 50$ fibers).

DECREASED POSTSYNAPTIC MUSCLE INPUT RESISTANCE ACCOUNTS FOR mEJP SIZE DECREASE IN HT-REARED LARVAE

The postsynaptic voltage responses, mEJPs and EJPs, reflect charging of muscle membrane capacitance by the synaptic currents, mEJCs and EJCs. The disparity between the HT-rearing effects on mEJP size and mEJC size prompted us to examine the passive electrical properties of the postsynaptic muscle fiber (i.e., muscle cell membrane resistance and time constant). To determine these parameters, we measured the voltage response to hyperpolarizing negative current injection of fixed amplitudes (-1 to -2 nA). The results showed a significant decrease in muscle input resistance in HT-reared larvae (**Figures 3A,B**). This indicates that when focal mEJCs remain unaltered, an increase in membrane conductance may be responsible for the diminished mEJPs in HT-reared larvae (**Figure 1B**). Indeed, we found a positive correlation between input resistance and mEJP size among individual muscle fibers (correlation coefficient = 0.59, $p < 0.01$, data not shown). Furthermore, there was no clear indication of changes in the membrane time constants as evidenced by unaltered half decay time upon cessation of hyperpolarizing current injection (**Figure 3C**). The results suggest no changes in the passive muscle membrane properties (membrane resistance and capacitance per unit area).

Taken together, for the postsynaptic muscle fiber as a functional unit, the whole-cell EJP size is homeostatically maintained after HT rearing. In spite of the increase in synaptic bouton numbers leading to the increase in quantal content ([EJP]/[mEJP]), stable synaptic strength is maintained through the counterbalancing effect of the decrease in postsynaptic muscle input resistance.

A ROLE OF *rut* AC IN SYNAPTIC HOMEOSTASIS: DIMINISHED HT-REARING EFFECTS ON MUSCLE INPUT RESISTANCE AND QUANTAL EVENTS

Previous *Drosophila* studies have established a clear role of cAMP in synaptic overgrowth induced by HT rearing or hyperexcitability mutations because mutations in *rut* AC suppress NMJ arborization or neuronal branching under such conditions (Zhong and Wu, 2004; Peng et al., 2007; Lee and Wu, 2010). Therefore we first examined the muscle input resistance of *rut* mutant larvae to determine whether the cAMP pathway is also involved in the HT-induced decrease in muscle input resistance. We observed a clearly different response to HT rearing in *rut* compared to WT larvae. When comparing RT-reared and HT-reared *rut* larvae, the muscle input resistance and membrane time constant remained unaltered, as evidenced by the similar responses to the same hyperpolarizing current injection (**Figures 3B,C**).

The above results prompted us to compare the sizes of mEJCs in RT- and HT-reared *rut* larvae (**Figure 4**) using loose-patch focal recording. We analyzed the same sets of focal recording data from 1997–2000 (Ueda and Wu, 2012) in parallel with the analysis for WT larvae (**Figure 2**). The results showed that the quantal unit of transmission, focal mEJCs (**Figure 4B**), was not altered by HT rearing in *rut* larvae, similar to the observation in WT larvae. However, synaptic efficacy in *rut* is drastic reduced by HT rearing, as reflected by diminished focal EJCs (**Figure 4A**; Ueda and Wu, 2012).

Based on the fact that the bouton number in *rut* larvae remains unchanged following HT rearing (Zhong and Wu, 2004), one should be able to predict the properties of EJPs in *rut* larvae following HT rearing. As a functional unit, the *rut* muscle fiber would display an unaltered size of quanta (mEJPs) but greatly diminished EJPs coupled with a reduction in quantal content. Our intracellular recording confirmed these predictions. Although the *rut* EJP amplitude was not significantly different from WT larvae when reared at RT, *rut* EJPs drastically reduced in size following HT rearing, in striking contrast to the homeostatic maintenance of EJPs in WT (Compare **Figures 1A** and **5A**). Furthermore, following HT rearing the mEJP size remained the same in *rut*, unlike a reduction in WT (Compare **Figures 1B** and **5B**). Correspondingly, HT rearing exerted opposite effects on the quantal content in *rut* (decrease) vs. WT (increase) (Compare **Figures 1C** and **5C**).

Finally, an examination of the spontaneous mEJP frequency revealed another transmitter release defect caused by *rut* upon temperature challenges. While HT rearing did not change the mEJP frequency in WT, there was a drastic decrease in spontaneous mEJP frequency in *rut* (Compare **Figures 1D** and **5D**). More extensive temperature-dependent defects in *rut* were also evident in short-term activity-dependent plasticity following HT rearing. Using a synaptic augmentation protocol (Zhong and Wu, 1991; Renger et al., 2000) with

high-frequency nerve stimulation (15 Hz, 10 s, 0.2 mM Ca^{2+}), *rut* larvae displayed drastically increased augmentation following HT rearing, whereas WT larvae retained the same augmentation properties. Therefore, short-term synaptic plasticity is also maintained in HT-reared WT larvae but disrupted in *rut* larvae with a striking enhancement of activity-dependent synaptic facilitation following HT rearing (**Figure 6**).

Our previous study has manipulated the expression of *rut* AC separately in pre- and post-synaptic compartments to examine the consequences on *rut* morphological phenotypes. With targeted expression of *UAS-rut*$^+$ by neuron- or muscle-specific *Gal4* drivers, it has been shown that the HT-induced NMJ overgrowth can be rescued by neuronal, but not muscular, expression of *rut*$^+$ (Zhong and Wu, 2004). Here we asked how pre- or post-synaptic expression of *rut*$^+$ modifies the defective synaptic homeostasis in HT-reared *rut*^{1} larvae. To express *rut*$^+$ in motor neurons and muscles in *rut*^{1} mutant background, we drove *UAS-rut*$^+$ expression by *C164-Gal4* (Torroja et al., 1999) and *mef2-Gal4* drivers (Ranganayakulu et al., 1996), respectively. We found that neither driver could totally rescue the *rut*^{1} phenotypes. Nevertheless, overexpression of *rut*$^+$ in muscle restored one aspect of the HT-induced homeostasis, i.e., EJP amplitude adjustment, but it did not modify muscle input resistance to mimic the WT response. In contrast, presynaptic overexpression of *rut*$^+$ in motor neurons did not rescue either of the HT-induced homeostasis.

For EJP amplitudes, muscle expression of *rut*$^+$ appeared to rescue the homeostatic maintenance (15.4 ± 4.2 and 16.6 ± 4.7 mV, $n = 8$ and 7 fibers, respectively, for RT and HT rearing, $p > 0.05$ with *t*-test), whereas expression of *rut*$^+$ in motor neurons lead to no restoration (21.6 ± 6.0 and 12.9 ± 7.7 mV, $n = 7$ and 7, $p < 0.05$). For the case of muscle input resistance, neither neuronal or muscle expression of *rut*$^+$ could rescue the expected HT-induced decrease, as seen in WT (neuronal expression: 7.8 ± 3.3 and 8.5 ± 2.5 Mohm, $n = 13$ and 15, $p > 0.05$; muscle expression: 7.3 ± 1.9 and 6.5 ± 2.0 Mohm, $n = 15$ and 15, $p > 0.05$).

DISCUSSION

SYNAPTIC HOMEOSTASIS AT THE *DROSOPHILA* NMJ

The *Drosophila* larval NMJ has been widely used for studying the genetic and molecular mechanisms underlying synaptic transmission and plasticity. Our study highlights a distinct category of synaptic homeostasis in response to environmental temperature stress. Our finding also indicates the involvement of the cAMP pathway in this form of homeostatic adjustment, further illustrating the important role of cAMP previously implicated in the various forms of synaptic and behavioral plasticity in *Drosophila* (Engel and Wu, 2009; Bushey and Cirelli, 2011; Kahsai and Zars, 2011; Ueda and Wu, 2012).

The first documented case of synaptic homeostasis at the *Drosophila* larval NMJ originates from a reduction in synaptic bouton numbers in mutants of the cell adhesion molecule Fasculin II (Stewart et al., 1996). This severe developmental alteration of the NMJ size nevertheless does not change the EJP amplitude, due to a compensatory increase in neurotransmitter release from individual synaptic boutons. Subsequent studies based on manipulations to decrease the postsynaptic quantal response further demonstrate the robust homeostatic adjustment in transmitter release at larval NMJs. Compensatory increases in the number of synaptic vesicle release (i.e., quantal content) occurs when the mEJP amplitude (quantal size) is diminished following either mutational disruption (Petersen et al., 1997; DiAntonio et al., 1999) or pharmacological blockade (Frank et al., 2006) of the postsynaptic glutamate receptors. In addition, when the mEJP amplitude is diminished due to a decrease in muscle input resistance by forced expression of inward rectifier K^+ channels (Kir; Baines et al., 2001), synaptic vesicle release from individual boutons is also homeostatically upregulated to maintain a stable EJP amplitude (Paradis et al., 2001).

A mechanism of retrograde trans-synaptic signaling via yet to be identified postsynaptic factors has been implicated in further studies. However, these studies also indicate that bone morphogenesis protein (BMP), encoded by *glassbottom boat* (*gbb*), is required for synaptic homeostasis (Goold and Davis, 2007; Frank et al., 2009). The action of BMP during the early phases of embryonic and larval development enables the HT- and hyperexcitability-induced synaptic growth that occurs at later stages of larval development (Berke et al., 2013). In addition, a number of presynaptic proteins also participate in the regulation of synaptic homeostasis, including Ephexin, a Rho-type guanine nucleotide exchange factor (Frank et al., 2009), *cacophony*-encoded voltage-gated calcium channels, (Frank et al., 2009; Müller and Davis, 2012; Lee et al., 2013), and a GTPase-activating protein Rab3 (Müller et al., 2011).

TEMPERATURE-DEPENDENT HOMEOSTATIC ADJUSTMENTS OF PHYSIOLOGICAL PARAMETERS IN MAINTAINING STABLE SYNAPTIC TRANSMISSION

Distinct from the above examples of synaptic homeostasis, our study reports a different category of synaptic plasticity upon long-term exposure to an environmental stressor, i.e., HT. This reflects an intrinsic ability of neuromuscular adaptation to ensure animal survival at stressful temperatures. Although the HT-induced increase in synaptic bouton numbers results in larger whole-cell synaptic currents (EJCs) (Sigrist et al., 2003), the EJP size recorded intracellularly in the postsynaptic muscle retains a level similar to that in RT-reared larvae (Ueda and Wu, 2012; **Figure 1**). Our data provide an explanation: A larger synaptic current does not lead to an increase in synaptic potential owing to a corresponding decrease in muscle input resistance.

Our results show that the postsynaptic current generated by each bouton actually remains unaltered following chronic HT exposure, as evidenced by constant sizes of the focal EJC and mEJC, indicating no changes in quantal content for the release from individual boutons (**Figure 2C**). The mEJP amplitude is nevertheless reduced due to a lower input resistance of the muscle membrane (**Figure 3**). Therefore, upon arrival of each action potential, the ensemble number of transmitter vesicles released from the axonal terminal is increased because of a

larger number of NMJ boutons, but this does not cause a larger postsynaptic muscle EJP due to a counterbalancing decrease in muscle input resistance. The simplest explanation is that long-term HT exposure induces the coordinated growth of both presynaptic nerve terminals and post-synaptic muscle such that a stable synaptic efficacy or muscle response is maintained. At present time, it is unclear whether the muscle growth precedes the nerve terminal ramification or the other way round during the process of HT-induced homeostatic adjustment, a topic that awaits further investigation.

These findings are largely consistent with previously publications (Sigrist et al., 2003; Berke et al., 2013), which reported NMJ overgrowth and increased EJC quantal content following HT treatment. However, it should be noted that in the voltage-clamp study of EJCs, Sigrist et al. (2003) did not report a decrease in the muscle input resistance of HT-reared larvae, i.e., the input resistance is about 6 Mohm, independent of rearing temperature. In contrast, our measurements based on current injection were 9.6 and 6.6 Mohm for RT- and HT-reared WT larvae, respectively (**Figure 3**). The reason for this difference is unknown. However, we note that the input resistance and mEJP amplitude for individual muscle fibers are highly variable, as evidenced from the ranges of data reported here (**Figures 1**, **3**). Thus, we base our conclusions on measurements from large samples of muscle fibers that met a stringent resting potential criterion to exclude muscles with membrane damage (see Section Materials and Methods).

Decreases in larval muscle input resistance have also been observed following acute heat treatments (Barclay and Robertson, 2003) or high-frequency nerve stimulation (Gertner et al., 2014), again highlighting the parallel between the effects of HT and hyperexcitability (Sigrist et al., 2003; Zhong and Wu, 2004). However, in these cases the input resistance decrease can be observed within a relatively short time period of a few tens of minutes, distinct from our long-term rearing effect following days of HT exposure. Acute heat-treatment results in decrease in both muscle input resistance and time constant (Barclay and Robertson, 2003), indicating no drastic changes in the total membrane capacitance (proportional to membrane area; Hille, 2001) of the muscle fiber. In contrast, our study does not indicate a HT rearing-induced change in membrane time constant (**Figure 3C**), reflecting an increase in effective membrane area without altering passive membrane properties (Hille, 2001) of the muscle fiber.

Interestingly, high-frequency (20 Hz) nerve activity is equally effective in decreasing larval muscle cell input resistance within a few tens of minutes (Gertner et al., 2014). Notably, this stimulus frequency is comparable to motor neuron firing rates during fictive locomotor activity in dissected larval preparations (Budnik et al., 1990; Fox et al., 2006; Chouhan et al., 2010). Also, the decline in the input resistance involves Ca^{2+}-dependent mechanisms since it is suppressed by genetic or pharmacological perturbations in a Ca^{2+}-activated K^+ (SK) channels or Ca^{2+}-dependent protein phosphatase 2A (Gertner et al., 2014). Interestingly, the *rut*^{1} mutation used in our study also abolishes the Ca^{2+}-dependence of AC activity (Dudai and Zvi, 1984; Livingstone et al., 1984).

RUT AC IN SYNAPTIC HOMEOSTASIS AND NEURAL PLASTICITY AT DIFFERENT TIME SCALES

We found that decreases in muscle input resistance following HT rearing were not observed in *rut* mutant larvae defective in AC, suggesting a role of cAMP in the homeostatic regulation mechanisms. In parallel, previous reports also document that *rut* mutations suppress HT-induced enhancement in neuronal growth, including dissociated neurons in culture (Peng et al., 2007), larval NMJ arbors (Zhong and Wu, 2004; Lee and Wu, 2010), and adult mushroom body neurons (Peng et al., 2007). These findings demonstrate the importance of cAMP signaling in the long-term adjustment of neuronal growth and function.

It should be noted that *rut* AC appears to be pervasively involved in different forms of activity-dependent neural plasticity, spanning a wide range of time scales, from seconds and minutes to hours and days. Within the range of seconds, activity-dependent, short-term plasticity of synaptic efficacy is well known to be altered by mutations of *rut* and *dunce* (*dnc*, encoding cAMP-specific phosphodiesterase), such as synaptic facilitation or depression at larval NMJs (Zhong and Wu, 1991; Renger et al., 2000; Ueda and Wu, 2009). Imaging studies of dissociated neurons in both *dnc* and *rut* cultures have also demonstrated altered growth cone motility (Kim and Wu, 1996) and abnormal Ca^{2+} transients in different neuronal compartments in the time frame of seconds (Berke and Wu, 2002). At more extended time scales of minutes, *dnc* and *rut* mutations affect activity-dependent recruitment of synaptic vesicles from the reserve pool (Kuromi and Kidokoro, 2000) and post-tetanic potentiation of transmitter release (Zhong and Wu, 1991) at the larval NMJ, as well as the habituation process of the jump-and-flight escape reflex mediated by the adult giant fiber (Engel and Wu, 1996).

With its wide ranging effects on activity-dependent neuronal plasticity, it is not surprising that the *rut* NMJs display detectable alterations in basic transmission properties. In addition to modified synaptic facilitation mentioned above (see also **Figure 6**), *rut* NMJs are known to have a range of alterations even at RT. For example, Ca^{2+} imaging demonstrates that influx through Ca^{2+} channels is more sensitive to Co^{2+} blockade; focal loose-patch recording reveals that vesicular release of transmitter appears to lack synchrony; and ultrastructural studies demonstrate a reduced number of docked vesicles and an increased area of synaptic density (Renger et al., 2000; Ueda and Wu, 2009). In the present study, we observed a tendency of increased frequency of spontaneous mEJP (compare **Figures 1D** and **5D**) and decreased augmentation (**Figure 6**) in *rut* larvae reared at RT. HT rearing further modifies some of these altered properties, including a reduction in mEJP frequency and a decrease in release efficacy, i.e., reduced quantal content (**Figure 5**).

For different forms of neuronal plasticity, the role of *rut* AC may vary in terms of the exact molecular mechanisms and cellular compartments of its action. As reported above, the Gal4-UAS experiments raise the possibility of *rut* AC involvement in pre- and post-synaptic interactions for the HT-induced synaptic homeostasis. We present below a comparison of hyperexcitability- and HT-induced synaptic homeostatic regulations that involve

a diversity of molecular players and cellular mechanisms orchestrated by cAMP signaling in the pre- and post-synaptic compartments.

HYPEREXCITABILITY- AND HT-INDUCED HOMEOSTATIC REGULATION OF SYNAPTIC FUNCTION DURING DEVELOPMENT

On the developmental time scale of hours to days, cAMP plays an important role in HT rearing- or hyperexcitability-induced overgrowth of nerve terminals. As described above, HT-rearing increases synaptic bouton numbers in WT larvae, which is suppressed by *rut* mutations (Zhong and Wu, 2004). Similarly, hyperexcitability mutations of various K^+ channels are known to promote nerve terminal overgrowth at the NMJ (reviewed in Fox et al., 2005). These K^+ channels include Eag or Kv10 encoded *by ether a-go-go* (*eag*, Warmke et al., 1991), Shaker or Kv1 encoded by *Shaker* (*Sh*, Kamb et al., 1987; Papazian et al., 1987; Pongs et al., 1988), Slo BK encoded by *slowpoke* (*slo*, Atkinson et al., 1991), and Erg or Kv11 encoded by *seizure* (*sei*, Titus et al., 1997; Wang et al., 1997). In many of these cases, the mutational effects on NMJ synaptic overgrowth are further enhanced by increased cAMP levels in *dnc* mutant backgrounds but suppressed by defective AC activity in *rut* mutant backgrounds (Zhong et al., 1992; Lee and Wu, 2010).

Importantly, HT rearing also further enhances the potency of NMJ overgrowth in several of these K^+ channel mutations, including *Sh* (Zhong and Wu, 2004) as well as *slo* and *sei* (Lee and Wu, 2010). The same is true for HT treatment on hyperexcitability-induced neurite overgrowth in the adult mushroom body, as well as cultured neurons of *eag* and *Sh* mutants (Peng et al., 2007). Our findings of the HT rearing-induced decrease in muscle input resistance accompanying synaptic bouton growth adds another dimension of the *rut* AC action on homeostatic functional matching between the pre- and post-synaptic elements.

The apparent parallel between the effects of hyperexcitability and HT rearing may be deceptive in terms of their underlying molecular and cellular mechanisms, even though *rut* AC plays important role in both cases. The hyperexcitable *slo* mutants have been studied in great detail for their pre- and post-synaptic readjustments to attain synaptic stability (Lee et al., 2008). Presynaptic terminals in *slo* mutants lack the critical regulation by Ca^{2+}-activated K^+ (BK) channel to terminate Ca^{2+} influx for transmitter release. However, EJPs in *slo* NMJs are nearly normal. Therefore, it may be instructive to contrast the two types of synaptic homeostatic mechanisms, one induced by HT rearing and the other by hyperexcitability, to illustrate their distinct homeostatic adjustments to pre- and post-synaptic components (**Figure 7**).

Figure 7A illustrates the pre- and post-synaptic homeostasis mechanisms induced by *slo* BK channel mutations to highlight the corresponding physiological readjustments for achieving stability of synaptic transmission (Lee et al., 2008). The surprising finding of nearly normal EJP sizes in *slo* mutants reveals a compensatory upregulation of a 4-AP sensitive K^+ current (likely Shaker I_A) for the defective BK currents. In addition, a re-composition of postsynaptic transmitter receptor GluRII (ratio between A and B

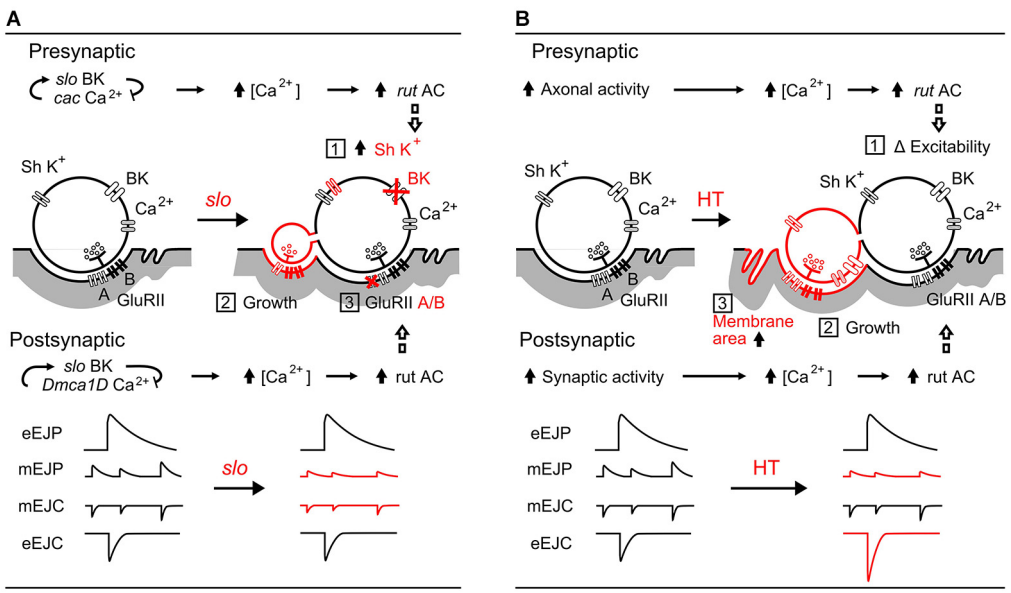

FIGURE 7 | Comparisons between *slo* hyperexcitabilty mutation- and HT rearing-induced synaptic homeostasis. **(A)** *slo* mutation-induced pre- and post-synaptic homeostatic adjustment (Lee et al., 2008, 2013; Lee and Wu, 2010): (1) upregulation of presynaptic *Sh* I_A K^+ currents to compensate for the loss of *slo* BK currents, (2) growth of excessive, functional satellite boutons, and (3) altered GluRII receptor composition (subunit A vs. B ratio) resulting in decreased mEJP amplitude. The roles of *cac* and *Dmca1D* Ca^{2+} channels, as well as the *rut* AC-mediated adjustments are depicted. **(B)** HT rearing induced-homeostasis: (1) regulation of excitability by *rut* AC (Ueda and Wu, 2009), (2) increased number of boutons (Zhong and Wu, 2004; Berke et al., 2013), and (3) decreased muscle membrane resistance from increased effective muscle membrane area. The resultant modifications in amplitudes of mEJPs and EJPs are summarized. In both **(A)** and **(B)**, *rut* AC plays an important role in homeostatic regulation of both synaptic function and growth.

subunits) leads to reduced mEJP and mEJC sizes. Even though *slo* induces extensive overgrowth of functional satellite boutons, the combined effects of these two adjustments partially restore the EJP size (**Figure 7A**, Points 1, 2, and 3). It is important to note that one of the characteristic features of *rut* AC action is its dependence on activity-dependent Ca^{2+} influx (Dudai and Zvi, 1984; Livingstone et al., 1984), presynaptically through the *cac* Ca^{2+} channels and postsynaptically through *Dmca1D* Ca^{2+} channels (Lee et al., 2013). In *rut* mutants, neither pre- nor post-synaptic homeostatic adjustments occur (Lee et al., 2008).

In contrast, as shown in **Figure 7B**, the HT-rearing induced synaptic bouton overgrowth is counterbalanced by an increase in muscle membrane area. HT rearing does not change the properties of individual boutons, and hence does not cause alterations in mEJC size. However, mEJP size is decreased due to a decrease in muscle input resistance, presumably owing to an increase in membrane area. Although the increase in bouton numbers leads to more vesicle release (increased quantal content) and larger EJC size, it is counterbalanced by the decreased input resistance to produce a nearly normal sized EJP, the ultimate whole-cell functional parameter of neuromuscular transmission.

A number of issues still remain to be further investigated in terms of the down-stream targets of *rut* AC in these two cases of synaptic homeostasis. Several well-established molecular networks that function in the larval NMJ should facilitate the endeavor to work out the molecular framework for different cases of HT rearing- and hyperexcitability-induced synaptic homeostasis. It has been shown that cAMP-dependent protein kinase (PKA) mediates protein phosphorylation and plays a role in larval NMJ synaptic function, with mutations of PKA subunits partially reflecting *dnc* or *rut* defects (Renger et al., 2000). Furthermore, manipulations of cAMP response element binding protein CREB have been shown to control synaptic growth at larval NMJs (Davis et al., 1996; Schuster et al., 1996).

ACKNOWLEDGMENTS

The authors would like to thank Anthony McGregor for his assistance in the preparation of this manuscript. This work was supported by NIH grants GM088804 and GM080255.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 31 October 2014; accepted: 08 January 2015; published online: 02 February 2015.

*Citation: Ueda A and Wu C-F (2015) The role of cAMP in synaptic homeostasis in response to environmental temperature challenges and hyperexcitability mutations. Front. Cell. Neurosci. **9**:10. doi: 10.3389/fncel.2015.00010*

This article was submitted to the journal Frontiers in Cellular Neuroscience.

frontiers in Cellular Neuroscience

ORIGINAL RESEARCH
published: 26 March 2015
doi: 10.3389/fncel.2015.00107

A single-cross, RNA interference-based genetic tool for examining the long-term maintenance of homeostatic plasticity

Douglas J. Brusich[1†], *Ashlyn M. Spring*[1,2†] *and C. Andrew Frank*[1,3*]

[1] *Department of Anatomy and Cell Biology, Carver College of Medicine, University of Iowa, Iowa City, IA, USA,* [2] *Interdisciplinary Graduate Program in Genetics, University of Iowa, Iowa City, IA, USA,* [3] *Interdisciplinary Programs in Genetics, Neuroscience, and MCB, University of Iowa, Iowa City, IA, USA*

OPEN ACCESS

Edited by:
Jaichandar Subramanian, Massachusetts Institute of Technology, USA

Reviewed by:
Konrad Ernst Zinsmaier, University of Arizona, USA
Susan Tsunoda, Colorado State University, USA

****Correspondence:***
C. Andrew Frank, Department of Anatomy and Cell Biology, University of Iowa, Carver College of Medicine, 51 Newton Road, 1-661A BSB Iowa City, IA 52242, USA
andy-frank@uiowa.edu

†*These authors have contributed equally to this work.*

Received: *16 December 2014*
Accepted: *09 March 2015*
Published: *26 March 2015*

Citation:
Brusich DJ, Spring AM and Frank CA (2015) A single-cross, RNA interference-based genetic tool for examining the long-term maintenance of homeostatic plasticity. Front. Cell. Neurosci. 9:107. doi: 10.3389/fncel.2015.00107

Homeostatic synaptic plasticity (HSP) helps neurons and synapses maintain physiologically appropriate levels of output. The fruit fly *Drosophila melanogaster* larval neuromuscular junction (NMJ) is a valuable model for studying HSP. Here we introduce a genetic tool that allows fruit fly researchers to examine the lifelong maintenance of HSP with a single cross. The tool is a fruit fly stock that combines the *GAL4/UAS* expression system with RNA interference (RNAi)-based knock down of a glutamate receptor subunit gene. With this stock, we uncover important new information about the maintenance of HSP. We address an open question about the role that presynaptic Ca_V2-type Ca^{2+} channels play in NMJ homeostasis. Published experiments have demonstrated that hypomorphic missense mutations in the Ca_V2 α1a subunit gene *cacophony* (*cac*) can impair homeostatic plasticity at the NMJ. Here we report that reducing *cac* expression levels by RNAi is not sufficient to impair homeostatic plasticity. The presence of wild-type channels appears to support HSP—even when total Ca_V2 function is severely reduced. We also conduct an RNAi- and electrophysiology-based screen to identify new factors required for sustained homeostatic signaling throughout development. We uncover novel roles in HSP for Drosophila homologs of Cysteine string protein (CSP) and Phospholipase Cβ (Plc21C). We characterize those roles through follow-up genetic tests. We discuss how CSP, Plc21C, and associated factors could modulate presynaptic Ca_V2 function, presynaptic Ca^{2+} handling, or other signaling processes crucial for sustained homeostatic regulation of NMJ function throughout development. Our findings expand the scope of signaling pathways and processes that contribute to the durable strength of the NMJ.

Keywords: homeostatic plasticity, *Drosophila melanogaster*, neuromuscular junction, RNAi screening, synaptic transmission, Ca_V2 channels, cysteine string protein, phospholipase C beta

Introduction

Animal nervous systems continually face challenges to normal function. When encountering neuronal stress, the outputs of synapses and circuits must be kept within a physiologically appropriate range. This restriction requires the activity of homeostatic regulatory systems. Homeostatic plasticity is a conserved principle across metazoan nervous systems. This fact is demonstrated by studies examining nematode, insect, crustacean, and mammalian synaptic preparations (Perez-Otano and Ehlers, 2005; Marder and Goaillard, 2006; Turrigiano, 2008; Pozo and Goda, 2010; Davis, 2013; Davis and Müller, 2015). Few molecules required for homeostatic synaptic plasticity (HSP) have been categorized into coherent signaling pathways. This is a gap in knowledge that limits our understanding of how neurons and synapses maintain stable function.

The *Drosophila melanogaster* third instar larval neuromuscular junction (NMJ) is a superb synapse for studying the molecular underpinnings of HSP (Frank, 2014a). At the fruit fly NMJ, genetic and pharmacological manipulations can be used to decrease the sensitivity of postsynaptic glutamate receptors to single vesicles of glutamate (decreased quantal size) (Petersen et al., 1997; Frank et al., 2006; Frank, 2014a). Decreased quantal size triggers retrograde (muscle-to-nerve) signaling that drives increased neurotransmitter release (increased quantal content, QC). As a result of this homeostatic signaling process, normal levels of muscle excitation are maintained.

Robust NMJ regulation has been exploited in genetic screens to uncover molecules required for HSP. One approach employs acute application of the glutamate receptor inhibitor, philanthotoxin-433 (PhTox) on semi-intact NMJ preparations of Drosophila larvae (Frank et al., 2006). Using Drosophila mutants, this approach has uncovered factors required for a short-term induction of synaptic homeostasis at the NMJ (10 min PhTox treatment), including presynaptic Ca_V2-type Ca^{2+} channels (Frank et al., 2006; Müller and Davis, 2012), K_V potassium channels (Bergquist et al., 2010), epithelial sodium (ENaC) channels (Younger et al., 2013), BLOC-1 complex members (biogenesis of lysosome-related organelles complex-1) (Dickman and Davis, 2009), SNARE complex members (soluble N-ethylmalemide-sensitive factor attachment receptors) (Dickman et al., 2012), Rab3-GAP (Müller et al., 2011), RIM (Rab3 interacting molecule) (Müller et al., 2012), RIM binding protein (Müller et al., 2015), and secreted endostatin (Wang et al., 2014). Some of these proteins gate important presynaptic molecular events such as an increase in Ca^{2+} influx or an increase in the size of the readily releasable pool of presynaptic vesicles (Weyhersmüller et al., 2011; Müller and Davis, 2012; Müller et al., 2012; Younger et al., 2013). These presynaptic events mirror salient aspects of HSP in mammalian neurons (Murthy et al., 2001; Burrone et al., 2002; Zhao et al., 2011). Therefore, homeostatic processes at the Drosophila NMJ appear to target fundamentally conserved mechanisms that are discoverable by genetic approaches.

The aggregate research at the NMJ suggests overlapping (yet distinct) classes of molecules are required for the acute induction of HSP and the long-term maintenance of HSP (Frank, 2014a). However, acute application of PhTox misses notable factors needed for the continued expression of synaptic homeostasis throughout life, such as the Rho-type guanine exchange factor Ephexin (Frank et al., 2009), the pair-rule transcription factor Gooseberry (Marie et al., 2010), and the protein translation regulator Target of Rapamycin (TOR) (Penney et al., 2012). Alternative approaches are required to identify and elucidate signaling processes the NMJ employs to maintain faithful neurotransmission in response to chronic challenges met throughout development. Signaling processes needed for the prolonged developmental expression of synaptic homeostasis at the Drosophila NMJ could serve a similar function in higher organisms.

A null Drosophila *GluRIIA* glutamate receptor subunit mutation (Petersen et al., 1997) is valuable for characterizing molecules that work to maintain homeostatic plasticity for extended developmental time (Frank et al., 2009; Marie et al., 2010; Penney et al., 2012; Frank, 2014a). *GluRIIA* loss decreases quantal size, and the NMJ responds with a homeostatic increase in presynaptic release (Petersen et al., 1997; DiAntonio et al., 1999). Yet Drosophila *GluRIIA* mutations are not perfectly ideal for large-scale, high-throughput genetic approaches to identify homeostatic factors. Use of these mutations in screens requires generations of genetic crossing, recombination (depending upon the genomic location of the screened mutation to be tested), and the generation of homozygous double mutants. All of this work needs to be completed prior to conducting electrophysiological analyses. Partial impairment of *GluRIII* (also known as *GluRIIC*) gene function presents an alternate possibility. GluRIII is an essential glutamate receptor subunit; null *GluRIII* mutations are embryonic lethal, but *GluRIII* mutant animals can be rescued to viability with low levels of *GluRIII* gene expression (Marrus et al., 2004). Third instar larval *GluRIII* hypomorphs have decreased quantal size and quantal frequency, but evoked excitation is normal because of a homeostatic increase in presynaptic release (Marrus et al., 2004).

For this study, we constructed a *Drosophila melanogaster* stock that exploits partial *GluRIII* loss to study the long-term maintenance of HSP in a single genetic cross. The stock takes advantage of the *GAL4/UAS* expression system and RNA interference (RNAi)-based expression tools (Brand and Perrimon, 1993; Lee and Carthew, 2003; Dietzl et al., 2007; Ni et al., 2008, 2009). Using this stock, we address an open question regarding how Ca_V2/Cacophony Ca^{2+} channel function gates synaptic homeostasis at the NMJ. Prior studies (ours included), demonstrate that hypomorphic, missense mutations in *cacophony* (*cac*) block homeostatic plasticity (Frank et al., 2006, 2009; Müller and Davis, 2012). Yet here we show that strong knock down of *cacophony* (*cac*) gene function throughout life is not sufficient on its own to impair homeostatic plasticity. We also conduct an RNAi- and electrophysiology-based screen to identify new molecules required for the long-term maintenance of homeostatic plasticity. We document novel roles in synaptic homeostasis for proteins previously implicated in intracellular Ca^{2+} regulation, including Drosophila homologs of Cysteine String Protein (CSP), Phospholipase Cβ (Plc21C), and Gαq.

Materials and Methods

Drosophila Husbandry and Stocks

Fruit flies were reared in chambers with temperature control (29°C for RNAi screen; otherwise 25°C). w^{1118} (Hazelrigg et al., 1984) is utilized as a wild-type control unless otherwise indicated. Drosophila stocks carrying various mutant alleles, *GAL4* drivers, or *UAS*-driven transgenes were used. Stocks were either obtained from the Bloomington Drosophila Stock Center (BDSC, Bloomington, IN), the Vienna Drosophila RNAi Center (VDRC, Vienna, Austria), or directly from researchers who generated them. *UAS-RNAi* lines from VDRC (Dietzl et al., 2007) detailed in this manuscript include: *UAS-cac[RNAi]* (VDRC transformant #104168; $cac^{KK101478}$), *UAS-Csp[RNAi]* (VDRC #34168; $Csp^{GD10571}$), and *UAS-Plc21C[RNAi]* (VDRC #26558; $Plc21C^{GD11359}$). *Plc21C* TRiP *UAS-RNAi* lines (Ni et al., 2008, 2009) obtained from BDSC include: $Plc21C^{HMS0600}$, $Plc21C^{JF01210}$, $Plc21C^{JF01211}$. A transgenic wild-type *cac* expression construct is *UAS-cac-eGFP*^{786C} (Kawasaki et al., 2004). Classical mutant alleles and deficiency stocks include: $GluRIIA^{SP16}$ (Petersen et al., 1997), $Csp^{DG29203}$ (Wishart et al., 2012), $Csp^{EY22488}$ (BDGP gene disruption project, Bellen et al., 2004), cac^{S} (Smith et al., 1998), cac^{HC129} (Kulkarni and Hall, 1987), $Plc21C^{p60A}$ (Weinkove et al., 1999), *Df(2L)BSC4* (J. Deal and K. Cook, BDSC), $G\alpha q^{1370}$ (Kain et al., 2008), $G\alpha q^{f04219}$ and $G\alpha q^{221c}$ (Banerjee et al., 2006), and $G\alpha q^{28}$ (Yao and Carlson, 2010). *GAL4* drivers include *elaV(C155)-GAL4* (Lin and Goodman, 1994), *Scabrous-GAL4* (Budnik et al., 1996), and *BG57-GAL4* (Budnik et al., 1996).

We constructed *UAS-GluRIII[RNAi]* by PCR amplification from genomic DNA, introducing *Xba I* restriction sites, and cloning a tandem duplicated fold-back version of the PCR product into the *pUAST pWiz* vector (Lee and Carthew, 2003). For the PCR amplification step, the *GluRIII* gene PCR primers were as follows: CAF2L: 5′-TCGATCTAGAGATCCT CGAGCGAGGATGGACAGCGGA-3′ and CAF2R: 5′-TTATT CTAGATGATTATCTCGCCAATGATGC-3′. Standard injection procedures were used to generate *UAS-GluRIII[RNAi]* transgenic stocks. A *UAS-GluRIII[RNAi]* insertion on chromosome III was built into a screening stock with the full genotype: *elaV(C155)-GAL4; Scabrous-GAL4/CyO-GFP; BG57-GAL4, UAS-GluRIII[RNAi]/TM6B*. For simplicity, this screening stock is termed *T15* in the manuscript, and balancer chromosomes are not included in its description in the Results Section of the text.

Electrophysiology and Analysis

Wandering third instar larvae were selected for electrophysiological recordings. Sharp electrode recordings were taken from muscle 6 of abdominal segments 2 and 3, as previously described (Davis et al., 1998; Frank et al., 2006). Larvae were dissected in a modified HL3 saline: NaCl (70 mM), KCl (5 mM), MgCl2 (10 mM), NaHCO3 (10 mM), sucrose (115 mM = 3.9%), trehalose (4.2 mM = 0.16%), HEPES (5.0 mM = 0.12%), and $CaCl_2$ (0.5 mM, unless otherwise indicated). Data were collected using Axopatch 200B or Axoclamp 900A amplifiers (Molecular Devices, Sunnyvale, CA), digitized using a Digidata 1440A data acquisition system (Molecular Devices), and recorded with pCLAMP 10 acquisition software (Molecular Devices). For presynaptic nerve stimulation, a Master-8 pulse stimulator (A.M.P. Instruments, Jerusalem, Israel) and an ISO-Flex isolation unit (A.M.P. Instruments) were utilized to deliver 1 ms suprathreshold stimuli to the appropriate segmental nerve. The average spontaneous miniature EPSP (mEPSP) amplitude was quantified by measuring the amplitude of ∼100–200 individual spontaneous release events per NMJ (unless mEPSP frequency was too low for ∼1 min continuous recording, in which case all mEPSPs available were measured). The average per-NMJ mEPSP amplitudes were then averaged for each genotype. The average evoked EPSP amplitude was calculated for each NMJ. Quantal content (QC) was determined for each recording by calculating the ratio of average EPSP and average mEPSP amplitudes. Quantal contents were calculated for each recording and then averaged across NMJs of the indicated genotypes. Where indicated, quantal content was corrected for non-linear summation (Martin, 1955). Selected raw electrophysiological data are included as Supplementary Table 1.

Immunostaining

Third instar larvae were fileted in HL3 saline. Dissected animals were fixed for 3 min in Bouin's fixative (Ricca Chemical Company, Arlington, TX), washed using standard procedures, and incubated in primary antibodies overnight at 4°C. This was followed by additional washes and a 2-hour incubation in secondary antibody at room temperature. Staining was performed using the following primary antibodies: mouse anti-Synapsin (3C11) 1:50 (Developmental Studies Hybridoma Bank, University of Iowa—DSHB) (Klagges et al., 1996); rabbit anti-Dlg 1:30,000 (Budnik et al., 1996); mouse anti-Brp (nc82) 1:250 (DSHB); rabbit anti-GluRIII 1:4000 (Marrus et al., 2004). The following fluorophore-conjugated antibodies were also used (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA): goat anti-mouse-488 1:1000 (DyLight); goat anti-rabbit-549 1:2000 (DyLight); Alexa-647 goat anti-HRP 1:500. Larval preparations were mounted in Vectashield (Vector Laboratories, Burlingame, CA) and imaged at room temperature using Zen software on a Zeiss 700 LSM mounted on an Axio Observer.Z1 using an EC Plan-Neofluar 40X Oil DIC Objective (aperture 1.30) or an EC Plan-Apochromat 63x Oil DIC Objective (aperture 1.40) (Carl Zeiss Microscopy, Jena, Germany). For each experiment, experimental and control larval preps were stained in the same container, mounted on the same slide, imaged using identical acquisition settings, and analyzed using the same procedure and thresholds.

Image Analyses

Bouton numbers were quantified semi-automatically using the "Spots" function in Imaris x64 v7.6.0 (Bitplane, Zurich Switzerland). Boutons were counted using the anti-Synapsin channel with the XY diameter set at 3 μm. Active Zones were counted using the anti-Brp channel with an XY diameter of 0.3 μm. GluRIII clusters were counted using the anti-GluRIII channel with an XY diameter of 0.5 μm. The threshold was adjusted so that each bouton, active zone, or GluRIII cluster was recognized once. Any errors in automated counting were corrected by

hand to arrive at the final value. GluRIII levels and area were assessed using ImageJ 1.48s/Java 1.6.0_24 (64-bit) with Fiji plugins. Z-stack images were compressed using the maximum projection function; an ROI was generated from a mask of the HRP channel and used to define the synapse; a second ROI was hand drawn to exclude any non-synaptic structures in the image; a minimum threshold was set for each channel to eliminate background fluorescence and held consistent within each experiment; the Measure function was used to assess fluorescence intensity and area for each channel [Brp(488), GluRIII(549), HRP(647)]. For each NMJ, total GluRIII fluorescence intensity and synapse coverage was normalized to the synaptic area of the HRP channel and compared between the genotypes analyzed. Cluster area was calculated by dividing the total synaptic area of GluRIII by the number of GluRIII clusters for each synapse imaged.

RNA Extraction and Quantitative RT-PCR

For each genotype, 25–30 wandering third instar, female larvae were sorted in HL3 saline, collected on ice and rinsed in HL3 with excess saline pipetted off before homogenization. Larvae were homogenized in 200 μL TRIzol reagent (Ambion, Life Technologies) using disposable plastic micropestles. Total homogenate was then brought to 1 mL by addition of 800 μL TRIzol, tubes were vigorously inverted, and the resultant homogenate was frozen at −80°C until all genotypes were prepared. Samples were allowed to thaw on ice and incubated for 5 min at room temperature before proceeding with RNA isolation using the manufacturer's instructions with the following changes: Following isopropanol addition, samples were placed at −20°C for precipitation, and two ethanol wash cycles were used. Samples were quantified using a Nanodrop (Thermo Scientific). Reverse transcription was done using iScript (Bio-Rad) according to manufacturer's instructions using 1 μg of total input. Quantitative PCR (Bio-Rad CFX96) was done using SYBR Green reagent (Bio-Rad) with a 10 μL final reaction volume and technical replicates in triplicate. Final primer concentrations were 250 nM. For *cac*, exon spanning primers were designed with the following sequences: cac-F 5′-cgggaacgagagttgtacg-3′ and cac-R 5′-actggagatggcagtacacg-3′ (this study), and RpL32-F 5′-atgaccatccgcccagcatac-3′ and RpL32-R 5′-ctgcatgagcaggacctccag-3′ (PK Geyer Lab, University of Iowa). Primer piloting was done using stepwise dilutions of iScript product to verify PCR efficiency. Final quantification was done using iScript product diluted to 1:444.4 in the final PCR. Melt curve analysis verified presence of a single product for all reactions.

Statistical Analyses

Statistical significance was assessed either by Student's *T*-Test, comparing an experimental data set and a control data set, or One-Way ANOVA with Tukey's *post-hoc* test across multiple data sets, as appropriate. Specific p value ranges are given in the text, figures, and figure legends, with $p < 0.05$ marked as significant ($^{\#}p = 0.05$; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$). For some p-values that trend toward statistical significance ($0.05 < p < 0.1$), specific values are given as indicated. The values reported or plotted on bar graphs are mean ± SEM.

Results

Homeostatic Challenge: *GluRIII* Gene Knock Down by RNAi

Partial knock down of *GluRIII* gene function should induce a significant decrease in NMJ quantal size and a homeostatic increase in NMJ quantal content (Marrus et al., 2004). Using the Drosophila *pWiz* vector (Lee and Carthew, 2003), we created a transgene to target the *GluRIII* gene for functional knock down by RNAi (see Materials and Methods). From a *pWiz-UAS-GluRIII[RNAi]* clone, we generated *UAS-GluRIII[RNAi]* transgenic Drosophila stocks and crossed them to stocks harboring the muscle-specific *GAL4* driver, *BG57-GAL4* (Budnik et al., 1996) (**Figure 1A**). We then analyzed the larval cross progeny by electrophysiology to assess the efficiency of glutamate receptor subunit knock down.

Postsynaptically driven *BG57-GAL4 >> UAS-GluRIII[RNAi]* yields a robust homeostatic challenge to NMJ function (**Figures 1B,C**) (See Supplementary Table 1 for selected raw electrophysiological data throughout the manuscript). NMJ effects are similar to the published *GluRIII* hypomorphic condition (Marrus et al., 2004). Compared to controls, *BG57-GAL4/UAS-GluRIII[RNAi]* NMJs show drastically decreased quantal amplitude (mEPSP = 0.81 ± 0.04 mV for wild-type control vs. 0.46 ± 0.05 mV for *GluRIII* knock down, $p < 0.001$, *T*-Test) and frequency (4.2 ± 0.2 Hz for control vs. 0.8 ± 0.1 Hz for *GluRIII* knock down, $p < 0.001$, *T*-Test). Despite these decreases in spontaneous miniature neurotransmission, evoked neurotransmission is normal (EPSP = 33.2 ± 1.0 mV for control vs. 33.6 ± 2.7 mV for *GluRIII* knock down) because of a homeostatic enhancement of quantal content ($QC = 43.8 \pm 2.1$ for control vs. 76.6 ± 7.1 for *GluRIII* knock down, $p < 0.01$, *T*-Test) (**Figures 1B,C**).

By meiotic recombination, we placed a *UAS-GluRIII[RNAi]* transgene on chromosome III in *cis* with the *BG57-GAL4* driver. To increase the potential of this stock as a genetic tool to study NMJ homeostasis, we augmented it with two presynaptic *GAL4* drivers. We chose neuronal drivers *elaV(C155)-GAL4* (Lin and Goodman, 1994) and *Scabrous-GAL4* (Budnik et al., 1996). Multiple presynaptic drivers were used to enhance the efficiency of RNAi in neurons. For simplicity, we refer to the new stock incorporating all drivers as *T15* (trans-synaptic). The genotype of *T15* is *elaV(C155)-GAL4*; *Sca-GAL4*; *BG57-GAL4, UAS-GluRIII[RNAi]* (chromosomes X; II; III, balancer chromosomes omitted from this genotype). We also generated a control stock, containing the same *GAL4* drivers, but not the *UAS-GluRIII[RNAi]* transgene.

When *T15* females are crossed to wild-type males (herein *T15 × WT*, **Figure 2A**), the larval progeny exhibit starkly diminished NMJ quantal size and frequency, and a homeostatic increase in NMJ quantal content (**Figures 2B,C**). By contrast, when *GAL4* driver control females are crossed to wild-type males (herein *GAL4 Cont × WT*), the NMJ electrophysiological profile of larval progeny is largely similar to that of wild-type NMJs (**Figures 2B,C**). The *GAL4* drivers or genetic background may induce a slight increase in quantal size (**Figure 2B**). However, this increase is not statistically significant, and the data show that the

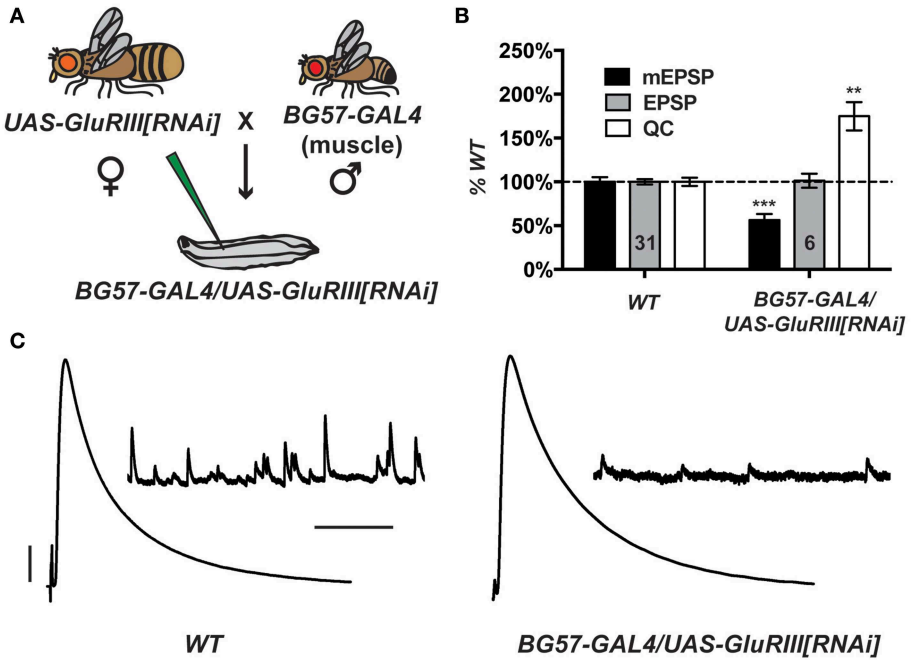

FIGURE 1 | Postsynaptic *GluRIII* gene knock down induces robust homeostatic compensation. (A) Crossing scheme. NMJs from F1 larvae (genotype *BG57-GAL4/UAS-GluRIII[RNAi]*) are subjected to electrophysiological analyses. **(B)** Quantal size (miniature excitatory postsynaptic potentials, mEPSP) is decreased for *BG57-GAL4/UAS-GluRIII[RNAi]* larvae (***$p < 0.001$, Student's *T*-Test). Evoked potentials (excitatory postsynaptic potentials, EPSP) are normal because of a homeostatic increase in quantal content (QC) (**$p < 0.01$). **(C)** Representative electrophysiological traces. Scale bars for EPSPs (mEPSPs): 5 mV (1 mV); 50 ms (1000 ms).

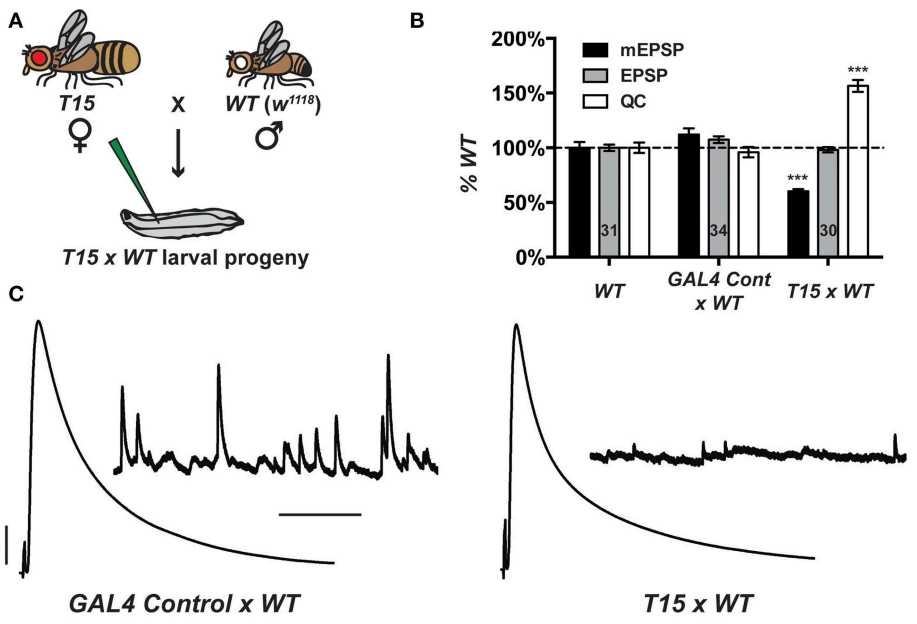

FIGURE 2 | The *T15* stock induces robust homeostatic compensation. (A) *T15* × *WT* crossing scheme. The genotype for *T15* is (chromosomes X; II; III): *elaV(C155)-GAL4; Scabrous-GAL4; BG57-GAL4, UAS-GluRIII[RNAi]*. **(B)** *T15* × *WT* larval NMJs have decreased quantal size (***$p < 0.001$, *T*-Test vs. *WT*). Evoked potentials are normal because of a homeostatic increase in QC (***$p < 0.001$). A control stock with only GAL4 drivers behaves similarly to *WT*. **(C)** Representative electrophysiological traces. Scale bars for EPSPs (mEPSPs): 5 mV (1 mV); 50 ms (1000 ms).

presence of presynaptic *GAL4* drivers exerts no adverse effects on evoked NMJ excitation (**Figures 2B,C**).

GluRIII Knock Down Does Not Grossly Alter Synapse Development

We wished to examine the effects of these NMJ drivers and the *UAS-GluRIII[RNAi]* transgene on synapse development. We imaged larval NMJs by immunofluorescence microscopy. We utilized an anti-GluRIII antibody to examine glutamate receptors (Marrus et al., 2004). As expected, *T15* × *WT* NMJs show a marked decrease in anti-GluRIII NMJ staining compared to wild-type controls or *GAL4* × *WT* controls (**Figures 3A–N**). We note several aspects of anti-GluRIII staining that are diminished at *T15* × *WT* NMJs. First, there is a 50% decrease in the number of anti-GluRIII puncta compared to wild-type control NMJs for muscles 6 and 7 (**Figure 3M**, $p < 0.05$, *T*-Test for both segments A2 and A3). In addition to this reduction in cluster number, we observed that individual GluRIII cluster size was greatly reduced ($41.6 \pm 3.5\%$ compared to wild-type controls, $p < 0.001$, *T*-Test). Further analysis of digital immunofluorescence images reveals that the average intensity of each anti-GluRIII pixel is also decreased ($82.3 \pm 3.9\%$ for the *T15* × *WT* condition compared to wild-type controls, $p = 0.03$, *T*-Test). This intensity decrease is small. However, when considered in combination with the reductions in cluster number and size, we estimate an 88% decrease in GluRIII NMJ protein per unit of synapse area in *T15* × *WT* larvae compared to wild-type larvae (**Figure 3N**, $p < 0.001$, *T*-Test; total fluorescence intensity, normalized for synapse area, see

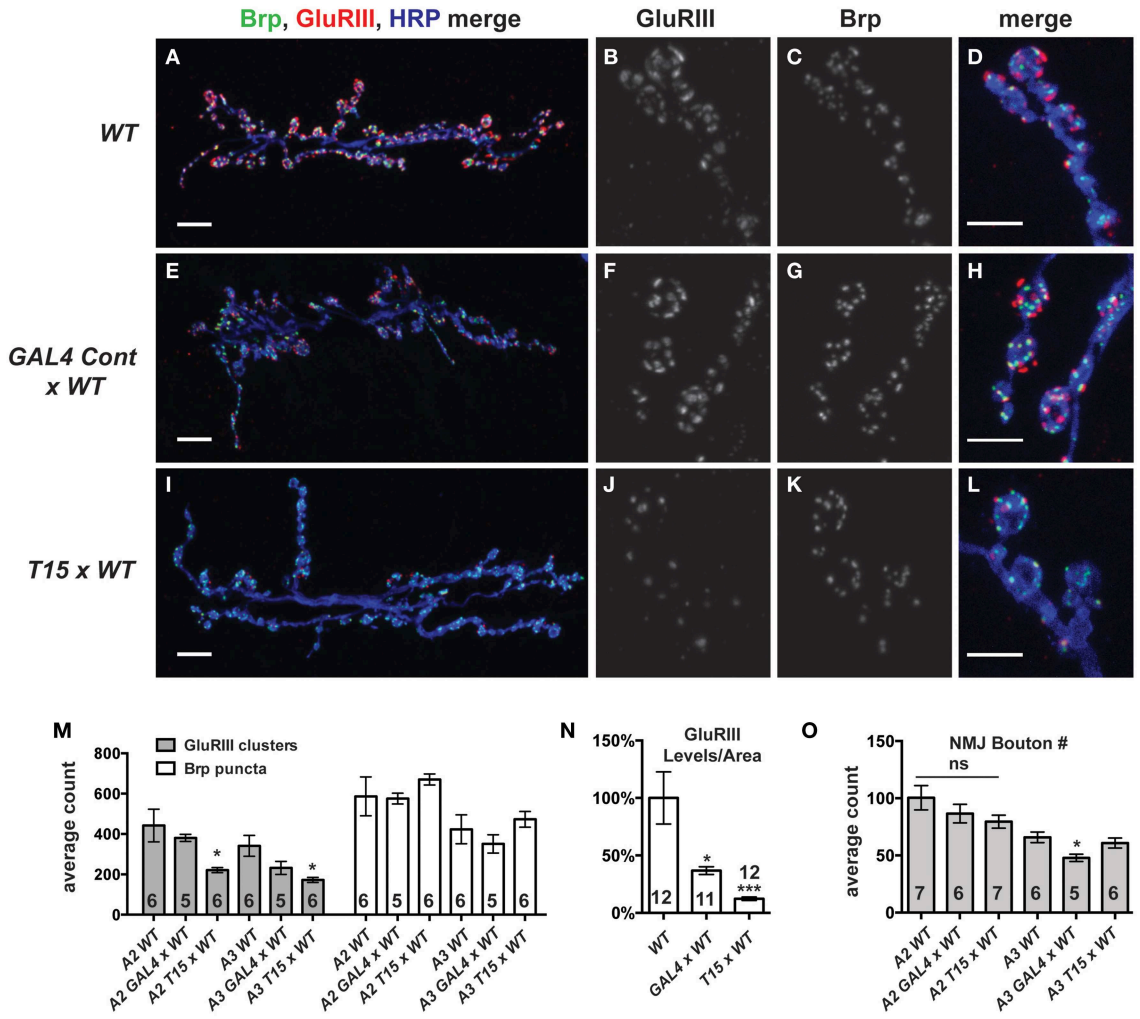

FIGURE 3 | GluRIII glutamate receptor subunits are dramatically decreased in the T15 line. (A-L) Immunostaining of wild type (*WT*), *GAL4 Cont* × *WT*, and *T15* × *WT* NMJs with antibodies against GluRIII (red), Bruchpilot (Brp, green), and HRP (blue). A, E, and I show 40X images (scale bars, 10 μm) of muscle 6/7 NMJs from wandering third instar larvae. **(B–D,F–H,J–L)** Panels show various channels of 60X images (scale bars, 5 μm) of NMJs. **(M)** Quantification of the number of presynaptic active zones (marked by Brp) and GluRIII clusters at the muscle 6/7 synapse of segments A2 and A3 (*$p < 0.05$). **(N)** Calculation of total GluRIII levels per unit of synapse area. This measure takes into account both GluRIII cluster size and GluRIII intensity (see text for individual values; see Materials and Methods for details; *$p < 0.05$ compared to WT; ***$p < 0.001$). **(O)** Quantification of the number of boutons at segment A2 and A3 muscle 6/7 NMJs. $n \geq 6$ NMJs stained for each condition.

Materials and Methods). As expected with RNAi-mediated knock down, this is not a complete loss of GluRIII protein. Importantly these changes are consistent with our electrophysiological data for *T15* × *WT* showing significantly decreased quantal size (reduced intensity/pixel and cluster size) and frequency (reduced cluster number).

Surprisingly, the presence of the *GAL4* drivers (or the genetic background of the driver line stock) also appears to diminish GluRIII protein level (per unit of synapse area) in *GAL4* × *WT* compared to wild-type controls (**Figure 3N**, $p < 0.05$). We are not certain why GluRIII levels are down in *GAL4* × *WT*. Nevertheless, by electrophysiology, this depression is clearly not severe enough to diminish quantal size or evoked excitation (**Figure 2B**)—though it could explain a partial decrease in quantal frequency compared to *WT* (Supplementary Table 1). Nevertheless, the amount of GluRIII protein per unit synapse area in *GAL4* × *WT* is about three times greater than the amount measured in *T15* × *WT* (**Figure 3N**, $p < 0.001$).

We wished to examine other basic elements of synapse structure. To do this, we stained NMJs with an anti-Bruchpilot (Brp) antibody to count the number of presynaptic active zones (Wagh et al., 2006) or with anti-Discs Large (Dlg) (Budnik et al., 1996) and anti-Synapsin (Syn) (Klagges et al., 1996) antibodies to count the number of synaptic boutons. For synapses on both segments A2 and A3, neither active zone counts (**Figure 3M**, Brp puncta) nor bouton counts (**Figure 3O**) are significantly different when comparing *T15* × *WT* NMJs vs. wild-type NMJs. These findings are interesting, considering the fact that *T15* × *WT* NMJs have fewer GluRIII clusters apposed to the presynaptic active zone (**Figure 3M**). Collectively, our data show that *T15* × *WT* NMJs have starkly decreased GluRIII levels, but otherwise grossly normal synapse growth and morphology.

RNAi- and Electrophysiology-Based Screening

The prior electrophysiological data illustrate the potential utility of *T15* as a genetic tool to study HSP. If *T15* animals are crossed to animals bearing an effective *UAS-RNAi* transgene, the target gene should be knocked down in larval progeny both presynaptically (due to *elaV(C155)-GAL4* and *Sca-GAL4* driving *UAS-RNAi* of the target gene in neurons) and postsynaptically (due to *BG57-GAL4* driving *UAS-RNAi* of the target gene in muscle)—all in the context of a homeostatic challenge to NMJ function due to *GluRIII* gene knock down. To employ *T15* for this purpose, we executed a small-scale RNAi- and electrophysiology-based screen. We chose 62 *UAS-RNAi* stocks on chromosomes II and III (Dietzl et al., 2007; Ni et al., 2008, 2009). The screen was biased: we targeted genes encoding factors potentially required for proper presynaptic Ca^{2+} entry and handling, G-protein signals (which could impinge upon calcium channel function), factors known to regulate general synaptic functions, and possible trans-synaptic signaling molecules. To conduct the screen, we crossed *T15* females with *UAS-RNAi* males and recorded from the NMJs of male larval progeny (**Figure 4A**). Male progeny were chosen because they should have a stronger dose of X-linked *elaV(C155)-GAL4* than female progeny.

T15 × *WT* NMJs have significantly elevated quantal content compared to non-transgenic wild-type controls (**Figures 2B**, **4B**). In analyzing the *T15* × *UAS-RNAi* screen data, we considered quantal content as a measure of presynaptic release. We identified eight *T15* × *UAS-RNAi* crosses that yielded progeny with a low NMJ quantal content ($QC < 52$, **Figure 4B**). For all eight, the level of presynaptic release is indistinguishable (or lower) than that of unchallenged, wild-type NMJs, potentially indicative of a block in homeostatic compensation. Considering the distribution of QC for all of the *UAS-RNAi* crosses from the screen, this level of presynaptic release is also about two standard deviations smaller than *T15* × *WT* controls. We wished to ensure that our synaptic release analysis was accurate and restrictive. Therefore, we also applied a correction to our calculations of quantal content to account for effects of non-linear summation (NLS) when recording synaptic voltages (Martin, 1955). Considering the distribution of NLS corrected QC for all of the *UAS-RNAi* crosses from the screen, an NLS corrected $QC < 85$ is two standard deviations smaller than *T15* × *WT* controls. Twelve *T15* × *UAS-RNAi* crosses fall below this NLS QC threshold, including all eight identified by the prior cutoff (**Figure 4C**). We chose to focus initial follow-up efforts on the eight potential positives that showed a low quantal content by both criteria (**Figure 4D**).

We identified eight potential hits out of 62 *UAS-RNAi* stocks, which is a very high positive rate for a screen (12.9%). However, the screen is biased, and it is not surprising that several pre-selected factors could impair synapse function. Nevertheless, most *T15* × *UAS-RNAi* crosses do not yield progeny with diminished NMJ QC. Several screen negatives with EPSP > 30 mV and $QC > 60$ are shown (**Figure 4E**). It is possible the corresponding target genes are not involved in synaptic homeostasis. However, it is also possible that conditions in this experiment do not yield sufficient gene knock down to reveal a homeostatic phenotype. One interesting example to consider in this regard is Drosophila *ephrin*. Prior work demonstrates that a presynaptic signaling system consisting of the Eph receptor tyrosine kinase and the cytoplasmic guanine exchange factor Ephexin is needed for the long-term maintenance of HSP (Frank et al., 2009). Drosophila Ephrin is a top candidate ligand for Drosophila Eph in this process, but the RNAi data from our screen do not support this conclusion (**Figure 4E**).

A low QC value from a screened *T15* × *UAS-RNAi* line could reflect a genuine defect in synaptic homeostasis. It could also simply reflect a decrease in neurotransmission when the targeted gene is knocked down in NMJ tissues. We note that two of the eight *UAS-RNAi* lines identified appear to cause severe neurotransmission defects (**Figures 4B–D**), suggesting synapse dysfunction independent of possible defects in homeostatic plasticity ($QC < 25$ when crossed to *T15*; NLS $QC < 35$ when crossed to *T15*). One of these two RNAi lines targets Drosophila *cacophony* (*cac*)—a positive control RNAi line included in the screen that targets the α1a pore-forming subunit of Ca_V2 channels. Given previous studies examining Ca_V2 in neurotransmission and NMJ homeostasis (Frank et al., 2006, 2009; Tsurudome et al., 2010; Müller and Davis, 2012), this result is not surprising. However, using *T15*, we are able to garner new information about Cac/Ca_V2 and its role in synaptic homeostasis (see below). Finally, we also report initial characterizations of two novel homeostatic genes, Drosophila *Csp* and *Plc21C* (**Figures 4B,C**).

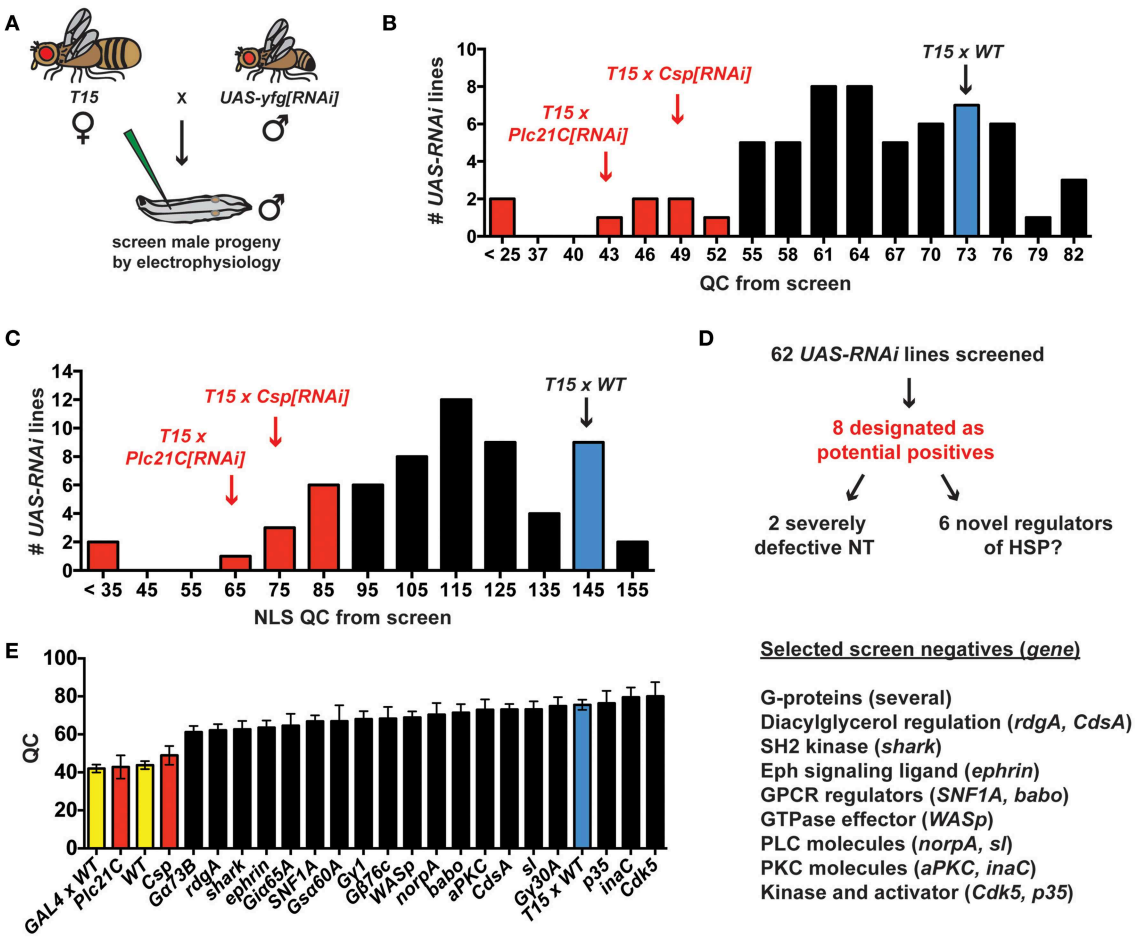

FIGURE 4 | An RNAi- and electrophysiology-based screen for homeostatic factors. (A) Crossing scheme for screen *T15 × UAS-yfg[RNAi]* ("your favorite gene"). For *UAS-RNAi* lines on chromosomes II or III, male progeny are examined by electrophysiology because dosage compensated *elaV(C155)-GAL4/Y* male progeny should have a higher dose of presynaptic GAL4 than *elaV(C155)-GAL4/+* female siblings. **(B)** Distribution of QC values from screened larvae. Eight *T15 × UAS-RNAi* crosses yield a QC smaller than two standard deviations below *T15 × WT* (red bars). **(C)** When QC is corrected for non-linear summation (NLS), twelve *T15 × UAS-RNAi* line crosses yield an NLS corrected QC (NLS QC) smaller than two standard deviations below WT (red bars). **(D)** Schematic to sort potential positives for follow-up analyses. **(E)** Negative data for some genes in the screen. Where a specific gene name is listed, the data represent the QC for *T15 × UAS-yfg[RNAi]*. Underlying data for displayed screen negatives has average evoked potentials >30 mV (not shown, but *WT* control EPSP = 33.2 ± 1.0 mV) and *QC* > 60.

Our screening and characterizations of other genes are ongoing, and we will report further characterizations elsewhere.

Cacophony Knock Down by RNAi Impairs Neurotransmission but Not Homeostatic Plasticity

Ca_V2-type voltage-gated Ca^{2+} channels are critical for presynaptic neurotransmission. For many vertebrate and invertebrate synapses, Ca_V2 channels also gate homeostatic modulations of neurotransmission (Frank, 2014b). In Drosophila, homozygous, partial loss-of-function missense mutations like cac^S block the homeostatic potentiation of transmitter release at the NMJ after glutamate receptor impairment (Frank et al., 2006, 2009; Müller and Davis, 2012). By contrast, *cac* null mutants arrest as embryos (Kulkarni and Hall, 1987; Kurshan et al., 2009). Therefore, it is unknown if *cac* mutant impairment of HSP is due to partial loss of channel function—or if amino-acid substitutions such as the one encoded by cac^S (F1029I, transmembrane domain III S6) (Smith et al., 1998) impair specific functions or interactions critical to homeostatic signaling. Intriguingly, $cac^S/+$ heterozygotes display partial defects in courtship song behavior (Smith et al., 1998) and the execution of synaptic homeostasis (Frank et al., 2006, 2009). These data are consistent with the possibility that missense *cac* mutations may exert antimorphic effects on synaptic function.

Knocking down *cac* gene activity by RNAi allows one to test whether a simple reduction of Ca_V2 function suffices to impair synaptic homeostasis. Efficient RNAi-mediated *cac* knock down also circumvents possible antimorphic effects associated with genetic mutations (but not potential effects due to haploinsufficiency). For the screen and subsequent analyses here, we used a *UAS-cac[RNAi]* transgene to knock down *cac* gene function

($cac^{KK101478}$, see Materials and Methods) (Dietzl et al., 2007). By primary target sequence, $cac^{KK101478}$ is not predicted to induce off-target gene knock down. By quantitative RT-PCR (qPCR), we find that driving this transgene under simultaneous control of the neuronal drivers *elaV(C155)-GAL4 and Sca-GAL4* diminishes total levels of *cac* mRNA by ∼50% vs. a control utilizing just the drivers (See Materials and Methods).

We prepared mRNA for qPCR using whole larvae. Therefore, Cac protein loss in motor neurons and at synapses could be even greater than qPCR measurements. We tested the effectiveness of *UAS-cac[RNAi]* on *cac* gene function in two different ways. First, we crossed transgenically rescued *cac* null females *elaV(C155)-GAL4,* cac^{HC129};; *UAS-cac-eGFP*^{786c} × *UAS-cac[RNAi]* males (or *WT* males for control). This *UAS-cac[RNAi]* cross (but not the *WT* control cross) kills all progeny, with only a small number of adults reaching the pupal eclosion stage and then either arresting during the process of eclosion or getting stuck in the food shortly after eclosing (not shown). Second, we find that presynaptic expression of $cac^{KK101478}$ starkly diminishes evoked neurotransmission (*elaV(C155)-GAL4, Sca-GAL4 >> UAS-cac[RNAi]*: EPSP = 8.5 ± 1.1 mV; $QC = 8.4 \pm 1.5$, $n = 8$) compared to a wild-type control (WT: EPSP = 33.2 ± 1.0 mV, $QC = 43.8 \pm 2.1$, $n = 31$). This diminishment of evoked neurotransmission is similar in severity to a cac^{S} mutant (Frank et al., 2006), and it suggests a significant loss of Cac protein at the NMJ. By contrast, muscle expression of $cac^{KK101478}$ (*BG57-GAL4 >> UAS-cac[RNAi]*) has no adverse effect on evoked neurotransmission (EPSP = 37.5 ± 2.0 mV; $QC = 53.2 \pm 2.9$, $n = 8$).

We crossed either the *T15* stock or the aforementioned pre- and post-synaptic *GAL4* driver control stock (**Figure 2**) to *UAS-cac[RNAi]*. For *GAL4 Cont × UAS-cac[RNAi]* progeny, NMJ evoked excitation and quantal content are markedly decreased compared to *GAL4 Cont × WT* controls (**Figure 5A**). This is true across a range of extracellular Ca^{2+} concentrations, though perhaps a bit less pronounced at physiological [Ca^{2+}] (1.5 mM) (**Figure 5A**). As expected, for *T15 × UAS-cac[RNAi]* larval progeny, there is a significant decrease in NMJ quantal size (mEPSP) compared to *GAL4 Cont × UAS-cac[RNAi]* larval progeny (**Figure 5B**). However, *T15 × UAS-cac[RNAi]* NMJ EPSP amplitude is no different than control cross *UAS-cac[RNAi]* progeny (**Figure 5C**). This is due to a homeostatic enhancement in presynaptic quantal content (**Figure 5B**). This result holds for extracellular Ca^{2+} concentrations that permit a range of presynaptic release: 0.5, 1.0, and 1.5 mM. For each condition, homeostatic compensation of presynaptic release remains robust (**Figure 5B**).

Our data suggest diminishment of *cac* gene function itself is not sufficient to block homeostatic potentiation of function. Nor is extracellular calcium concentration a factor. If Cac/Ca_V2 levels are simply diminished—but the copies of Cac α1a subunits present are wild-type copies—homeostatic plasticity is intact. By contrast, the presence of function-impairing Ca_V2 α1a subunits due to amino-acid substitution appears sufficient to block the induction and maintenance of homeostatic plasticity (Frank et al., 2006, 2009; Müller and Davis, 2012).

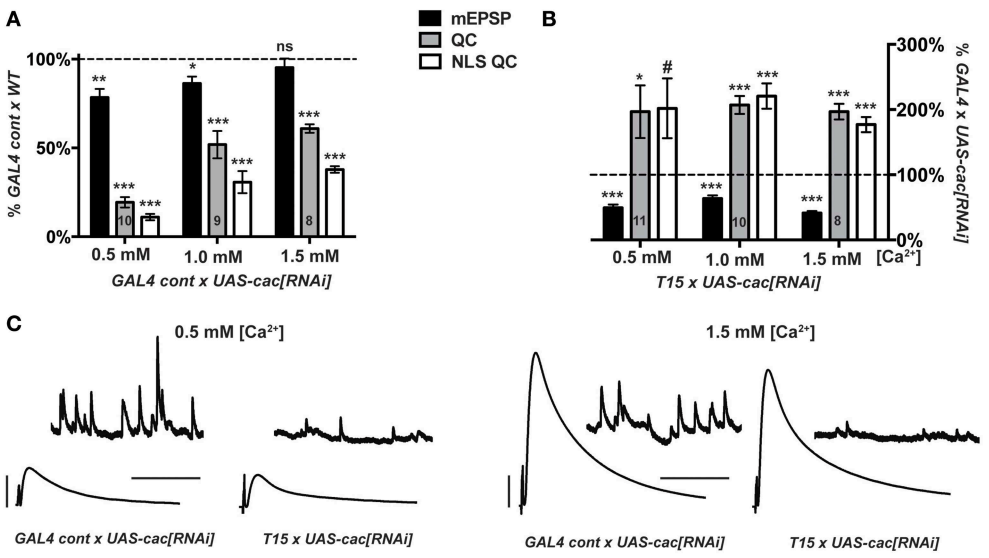

FIGURE 5 | Knock down of *cacophony* gene function does not impair synaptic homeostasis. (A,B) mEPSP (black), QC (gray), NLS QC (white). (#$p = 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, *T*-test compared to control at 100% dotted line) **(A)** Diminished baseline neurotransmission for *GAL4 control × UAS-cac[RNAi]* larvae is consistent with *cac* gene knock down. Diminished release occurs across a range of extracellular [Ca^{2+}]. **(B)** *T15 × UAS-cac[RNAi]* larvae show robust homeostatic compensation compared to *GAL4 control × UAS-cac[RNAi]* larvae. As expected, the *T15* line induces a marked diminishment of quantal size (mEPSP). The NMJ responds with a robust increase in release. This response is observed across a range of extracellular calcium concentrations, and the same result holds whether or not QC is corrected for non-linear summation. **(C)** Representative electrophysiological traces. Scale bars for EPSPs (mEPSPs): 5 mV (1 mV); 50 ms (1000 ms).

Cysteine String Protein Mutations Block the Long-Term Maintenance of Synaptic Homeostasis

CSP is a conserved synaptic protein in the DnaJ family of chaperones. For both vertebrates and invertebrates, CSP executes functions that promote viability, coordinated locomotion, neuroprotection, and evoked neurotransmitter release (Zinsmaier, 2010). At the Drosophila NMJ, synaptic functions of CSP appear related to Ca^{2+} regulation. In *Csp* loss-of-function mutants, resting intra-terminal Ca^{2+} levels are altered (Dawson-Scully et al., 2000; Bronk et al., 2001; Dawson-Scully et al., 2007), and neurotransmission defects can be suppressed by high $[Ca^{2+}]_e$ or repetitive nerve stimulation (Dawson-Scully et al., 2000; Bronk et al., 2001). The fact that a *UAS-Csp[RNAi]* line emerged from our screen was somewhat surprising because a prior genetic screen demonstrated that a *Csp* mutation does not impair homeostatic plasticity when the NMJ is challenged with PhTox application (Goold and Davis, 2007; Dickman and Davis, 2009). However, this previous screen specifically examined factors for a role in the short-term induction of homeostatic plasticity, not its long-term maintenance.

Our screen found no elevation of NMJ quantal content when comparing *T15 × UAS-Csp[RNAi]* larval progeny vs. wild-type larvae (**Figure 4**). This result indicates a possible role for CSP in homeostatic plasticity, although it could also be consistent with expected *Csp* defects in neurotransmission. In a follow-up test, we found that *T15 × UAS-Csp[RNAi]* NMJ quantal content is not significantly different than *GAL4* driver control × *UAS-Csp[RNAi]* NMJ quantal content (**Figure 6A**). This

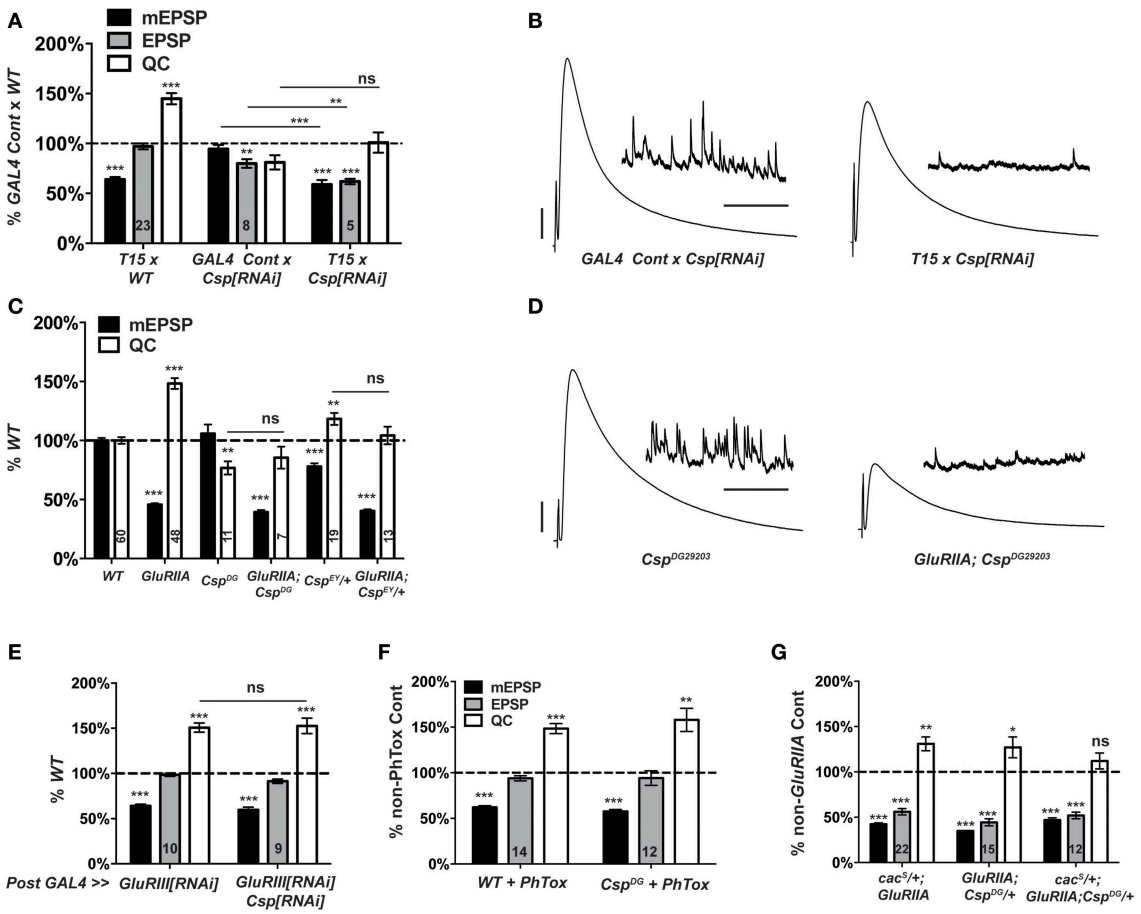

FIGURE 6 | C*sp* is required for long-term homeostatic compensation. **(A)** *T15 × UAS-Csp[RNAi]* shows a failure to upregulate quantal content compared to its *GAL4*-driven *UAS-Csp[RNAi]* control (ns, $p = 0.15$). Knock down of *Csp* shows a slight impairment in evoked neurotransmission (EPSP) compared to control ($^{**}p < 0.01$). **(B)** Representative electrophysiological traces show the failure of *T15 × UAS-Csp[RNAi]* larvae to maintain evoked potentials at control levels. **(C)** Homozygosity for the $Csp^{DG29203}$ allele or heterozygosity for the $Csp^{EY22488}$ allele block homeostatic upregulation of quantal content compared to their respective non-$GluRIIA^{SP16}$genetic controls (ns, $p = 0.44$ and $p = 0.14$, respectively). **(D)** Representative traces show a failure of homeostatic compensation for $GluRIIA^{SP16}$, $Csp^{DG29203}$. **(E)** Postsynaptic knock down of *Csp* function leaves homeostatic plasticity intact. **(F)** Acute homeostatic compensation is intact in $Csp^{DG29203}$ as evidenced by the elevated quantal content in response to philanthotoxin-433 (PhTox) application ($^{**}p < 0.01$). **(G)** A doubly heterozygous combination of $Csp^{DG29203}/+$ and $cac^S/+$ shows a homeostatic block in the $GluRIIA^{SP16}$ background because of a failure to increase quantal content over $cac^S/+$; $Csp^{DG29203}/+$ controls (ns, $p = 0.24$). By contrast, the single heterozygous mutations retain partial homeostatic compensatory capacity. Scale bars for EPSPs (mEPSPs): 5 mV (1 mV); 50 ms (2000 ms). (ns, $p > 0.05$; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

result suggests a bona fide defect in synaptic homeostasis (**Figures 6A,B**).

To follow up on the RNAi screen with genetic mutations, we examined two transposon insertion-induced *Csp* alleles, $Csp^{DG29203}$ and $Csp^{EY22488}$. $Csp^{DG29203}$ disrupts the 5′ UTR of *Csp*, and genetic evidence suggests that it is hypomorphic (Wishart et al., 2012). $Csp^{EY22488}$ is inserted in an intronic region of *Csp*. It causes significant homozygous lethality and appears to be a strong mutation genetically (data not shown). We generated two double mutant combinations: $GluRIIA^{SP16}$; $Csp^{DG29203}$, and *GluRIIA*; $Csp^{EY22488}$/+. The $GluRIIA^{SP16}$ deletion mutation induces a marked decrease in quantal size and a homeostatic increase in quantal content compared to wild-type controls (**Figure 6C**) (Petersen et al., 1997). By contrast, for the double mutant combinations, synaptic homeostasis is impaired: there is no significant increase in release compared to *Csp* mutants alone (**Figure 6C**). As a result, evoked postsynaptic excitation is stunted (**Figure 6D**). Interestingly, the $Csp^{EY22488}$/+ and $Csp^{DG29203}$ genetic conditions display somewhat different baseline electrophysiology, in terms of both quantal size and quantal content (**Figure 6C**). These differences could be due to different genetic backgrounds or *Csp* allelic strength. Nevertheless, both alleles block homeostatic compensation in response to *GluRIIA* gene loss (**Figures 6C,D**).

The vast majority of research published about Drosophila CSP demonstrates that it localizes to presynaptic terminals. However, it has also been reported that Drosophila muscles can express low levels of CSP (Eberle et al., 1998). To test whether CSP's function in synaptic homeostasis could reside in the postsynaptic compartment, we crossed the muscle driver stock *BG57-GAL4, UAS-GluRIII[RNAi]* to *UAS-Csp[RNAi]*. Knocking down *Csp* gene function in the muscle leaves synaptic homeostasis intact (**Figure 6E**); this result is consistent with a presynaptic role for CSP in synaptic homeostasis.

Collectively, our data demonstrate that *Csp* function is needed for the long-term maintenance of homeostatic plasticity. To double check whether *Csp* could also be required for the short-term induction of homeostatic plasticity, we applied PhTox to $Csp^{DG29203}$ mutant NMJs. By this assay, the rapid induction of homeostatic plasticity remains intact (**Figure 6F**), consistent with the previously reported results (Goold and Davis, 2007; Dickman and Davis, 2009). Therefore, CSP appears to be needed for the sustained expression of homeostatic plasticity throughout development, not its short-term induction.

A *Csp* and *cac* Mutant Combination Blocks HSP

cac^S/+; *GluRIIA* double mutant NMJs have partially impaired synaptic homeostasis (Frank et al., 2006) (and **Figure 6G**). As discussed above, this partial impairment could be due to an antimorphic allelic effect. The cac^S/+ genetic condition has been previously exploited to characterize second-site factors that could potentially execute homeostatic plasticity in conjunction with Ca_V2 by searching for genetic interactions with cac^S (Frank et al., 2009; Wang et al., 2014). In the sensitized cac^S/+ genetic background, we find that cac^S/+; *GluRIIA*; $Csp^{DG29203}$/+ NMJs have completely blocked synaptic homeostasis—i.e., there is no increase in quantal content compared to the cac^S/+;; $Csp^{DG29203}$/+ genetic control condition (**Figure 6G**). A strong double heterozygous phenotype could be consistent with Cac and CSP both functioning within synaptic homeostasis, either in a linear signaling pathway or in parallel processes. However, it could also reflect a summation of separate, partial defects in HSP for both cac^S/+; *GluRIIA* NMJs and *GluRIIA;* $Csp^{DG29203}$/+ NMJs (**Figure 6G**).

Loss of PLCβ Function Impairs Synaptic Homeostasis

In designing our screen, we postulated that lipid signaling at the synapse could affect NMJ homeostasis. Canonically, Gαq-GTP (together with Gβγ G-proteins) activates PLCβ. In turn, PLCβ cleaves phosphatidylinositol 4,5-bisphosphase (PIP_2) into diacylglycerol (DAG) and inositol triphosphate (IP_3) (Tedford and Zamponi, 2006; Philip et al., 2010; Kadamur and Ross, 2013). These molecules are known to influence neurotransmission in several ways at many synapses, including the NMJ (Goni and Alonso, 1999; Cremona and De Camilli, 2001; Peters et al., 2001; Wu et al., 2002; Rohrbough and Broadie, 2005; Huang et al., 2006). To examine this pathway in the context of NMJ homeostasis, we considered a Drosophila PLCβ homolog gene, *Plc21C*—among other genes, some of which showed no phenotype (**Figure 4E**).

When *T15* is crossed to a *UAS-Plc21C[RNAi]* transgenic line (Dietzl et al., 2007), larval progeny have significantly lower NMJ quantal content than *T15* × *WT* controls (**Figures 4B,C**, **7A**). By contrast, when the muscle driver stock *BG57-GAL4, UAS-GluRIII[RNAi]* is crossed to the same *UAS-Plc21C[RNAi]* transgenic line, full homeostatic compensation occurs (**Figures 7B,C**). This latter result argues against Plc21C acting in the muscle to control synaptic homeostasis. A prior study demonstrated that presynaptic expression of the same *UAS-Plc21C[RNAi]* construct utilized in our screen ($Plc21C^{GD11359}$) diminishes *Plc21C* mRNA levels by more than 50% (Dahdal et al., 2010). Moreover, *Plc21C* mRNA is found in the larval nervous system (Shortridge et al., 1991). Collectively, these data are consistent with a neuronal role for Plc21C in the homeostatic control of NMJ function.

To address possible off-target effects of *UAS-Plc21C[RNAi]* expression, we acquired three additional *UAS-Plc21C[RNAi]* lines—Drosophila TRiP constructs (Transgenic RNAi Project) (Ni et al., 2009, 2008). Each TRiP line fails to robustly increase quantal content when crossed to *T15*, (**Figure 7A**). This result is consistent with an impairment of HSP. Additionally, we examined two heterozygous *Plc21C* deletion mutations: a chromosomal deficiency that removes one copy of *Plc21C* from the genome, *Df(2L)BSC4*, and a 5′ UTR and first exon deletion called $Plc21C^{p60A}$ (Weinkove et al., 1999). Alone, neither heterozygous deletion diminishes evoked neurotransmission (**Figures 7C,D**). Heterozygous *Plc21C*/+ animals in a $GluRIIA^{SP16}$ mutant background do have a mild increase in quantal content compared to *Plc21C*/+ genetic controls (**Figure 7E**). However, this increase is not nearly as robust as one would expect for a *GluRIIA* condition (**Figure 7E**). This result indicates a partial impairment of NMJ homeostatic compensation (**Figures 7C,E**). Collectively, the data utilizing hypomorphic *Plc21C* conditions support the idea that

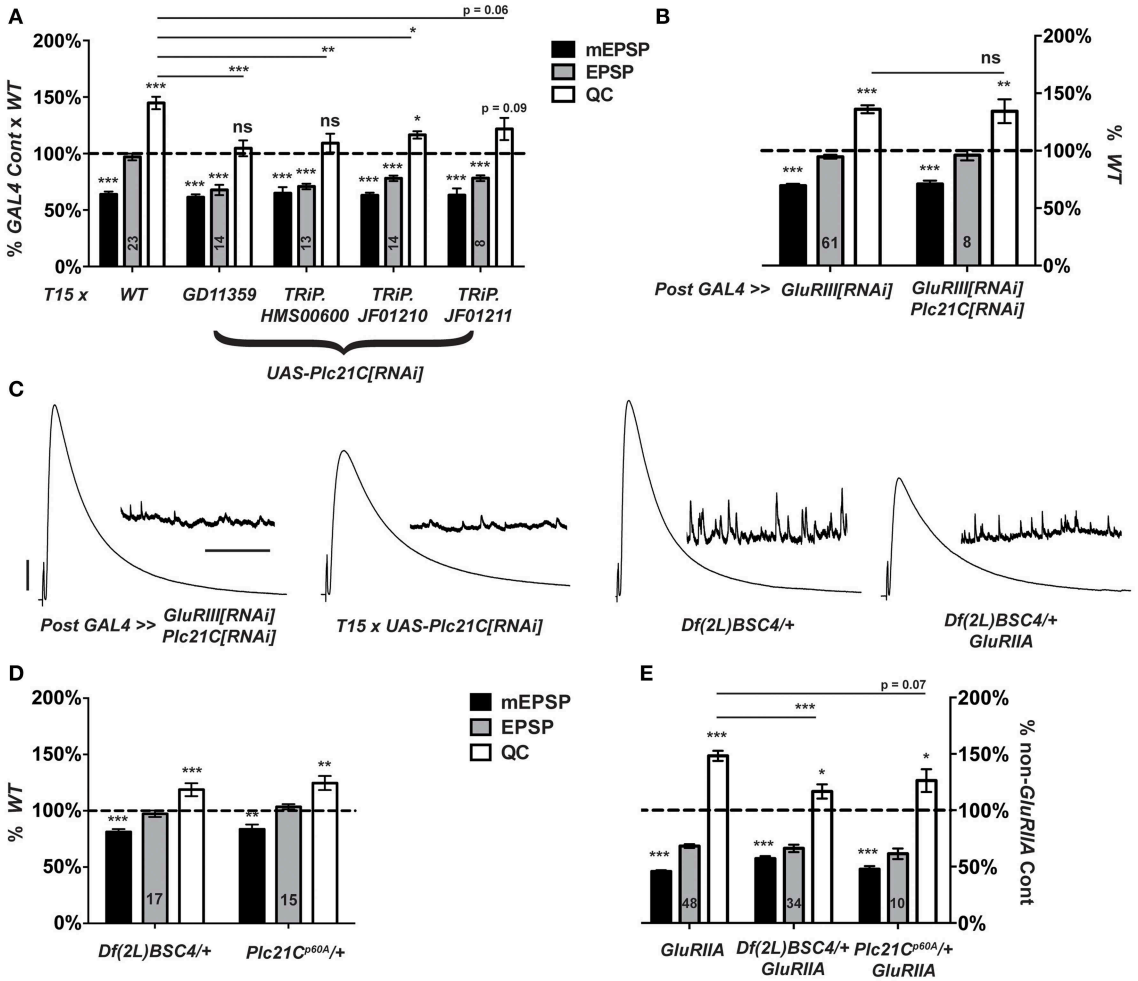

FIGURE 7 | Presynaptic *Plc21C* is required for homeostatic compensation. (A) *T15*-mediated RNAi knock down of *Plc21C* results in a failure to maintain the EPSP at control levels (***$p < 0.001$). Concerning homeostatic compensation, *T15* × *TRiP.JF01210* is the only cross to show increased quantal content compared to its *GAL4* cross control (*$p = 0.04$), but even this quantal content was still significantly depressed compared to the *T15* × *WT* control (*$p < 0.05$, One-Way ANOVA including all crosses in dataset, with Tukey's *post-hoc*). **(B)** Postsynaptic knock down of *Plc21C* does not affect homeostatic compensation: the EPSP is maintained (ns, $p = 0.45$) at control levels because the quantal content is upregulated (**$p < 0.01$). **(C)** Representative traces show normal neurotransmission upon postsynaptic knock down of *GluRIII* and *Plc21C* or a heterozygous loss of *Plc21C* via use of the deficiency chromosome *Df(2L)BSC4*. However, evoked release is not maintained upon *T15*-mediated knock down of *Plc21C* or the heterozygous loss of *Plc21C* in a *GluRIIA*^{SP16} mutant background. **(D)** Evoked amplitudes (EPSPs) are unaltered upon heterozygous loss of *Plc21C* by use of the deficiency *Df(2L)BSC4* or *Plc21C*^{p60A} allele though both do show a small, yet significant decrease in the amplitude of mEPSP events (***$p < 0.001$ and **$p < 0.01$, respectively). **(E)** Quantal content is minimally increased in the *GluRIIA*^{SP16} background upon heterozygous loss of *Plc21C* compared to their respective genetic controls (*$p < 0.05$). For the *Df(2L)BSC4* deficiency, the increased quantal content does not reach the level of increase found in *GluRIIA*^{SP16} (***$p < 0.001$, One-Way ANOVA, Tukey's *post-hoc*). This is indicative of a partial impairment in homeostatic compensation. Scale bars for EPSPs (mEPSPs): 5 mV (1 mV); 50 ms (2000 ms).

presynaptic *Plc21C* gates the sustained expression of homeostatic plasticity.

A Role for Gαq in Synaptic Homeostasis?

Gαq-GTP and Gβγ G-proteins are classically known to activate PLCβ function (Tedford and Zamponi, 2006). A positive Gαq-Plc21C regulatory relationship appears conserved for Drosophila flight behavior (Banerjee et al., 2006). Therefore, we postulated that *Plc21C* could be playing a role in synaptic homeostasis downstream of G-protein signals. In the course of our screen, we discovered that genetic knock down of a Gαq-encoding gene only mildly impairs synaptic release compared to wild-type: *T15* × *UAS-Gαq[RNAi]* (EPSP = 29.8 ± 1.6 mV; QC = 64.0 ± 3.7). This effect was not strong (possibly due to partial gene knock down by RNAi) and was not identified as a positive hit from the screen. However, given the results with *Plc21C*, we wished to probe *Gαq* with genetic mutants. We examined four strong Gαq loss-of-function alleles: *Gαq*^{1370}, *Gαq*^{f04219}, *Gαq*^{221c}, and *Gαq*^{28}. Each *Gαq* null allele is homozygous lethal (well before the third instar larval stage), but *Gαq*/+ heterozygotes are viable. For baseline

neurotransmission, *Gαq*/+ mutant NMJs show normal levels of postsynaptic excitation (EPSPs, **Figure 8A**). Interestingly, however, heterozygous *Gαq*/+ NMJs show partially impaired homeostatic plasticity in a *GluRIIA*^{SP16} mutant background (**Figure 8B**).

Each *Gαq*/+ mutant condition shows a small, but significant decrease in NMJ quantal size compared to wild-type controls (**Figure 8A**). We wondered if this decrease in quantal size could reflect a decrease in glutamate receptor expression levels and a postsynaptic role for Gαq. This possibility could potentially confound our analyses of homeostatic plasticity. Therefore, we stained *Gαq*/+ mutant NMJs with anti-GluRIII and anti-GluRIIA antibodies and quantified stained receptor levels per unit synapse area, as before (**Figure 3**). We find no significant decrease in glutamate receptor levels in *Gαq*/+ mutant NMJs (**Figures 8C,D**). In fact, for most measures, there appears to be a slight increase or a statistical trend toward a slight increase of glutamate receptor subunit expression (**Figure 8C**). This single experiment does not rule out possible postsynaptic functions for Gαq. However, our collective findings are consistent with the possibility that Gαq and Plc21C play concerted roles in the execution of homeostatic plasticity. Further experiments will be required to test if there is a direct regulatory relationship in the presynaptic nerve between the two proteins.

Discussion

There exists overwhelming evidence that forms of HSP shape how neurons and synapses maintain stable function. Physiologically appropriate levels of synaptic activity must be maintained throughout life while facing numerous endogenous and exogenous challenges to neuronal function. To understand how HSP works on a molecular level, it is important to uncover mechanisms that govern both its initiation and long-term maintenance. Here, we apply prior knowledge and reagents to develop a new genetic stock abbreviated as *T15*: *elaV(C155)-GAL4; Sca-GAL4; BG57-GAL4, UAS-GluRIII[RNAi]*. *T15* allows examination of the long-term maintenance of synaptic homeostasis at the NMJ in a single genetic cross. We validate the synaptic properties of *T15* progeny through a combination of genetics, electrophysiology, and synapse imaging. We demonstrate the utility of *T15* as a genetic tool in multiple ways: (1) we address one aspect of how Ca_V2 function impinges upon synaptic homeostasis throughout development; (2) we identify CSP as a novel regulator of homeostatic plasticity; and (3) we identify a putative presynaptic system consisting of Drosophila Gαq and PLCβ homologs that also regulates homeostatic plasticity.

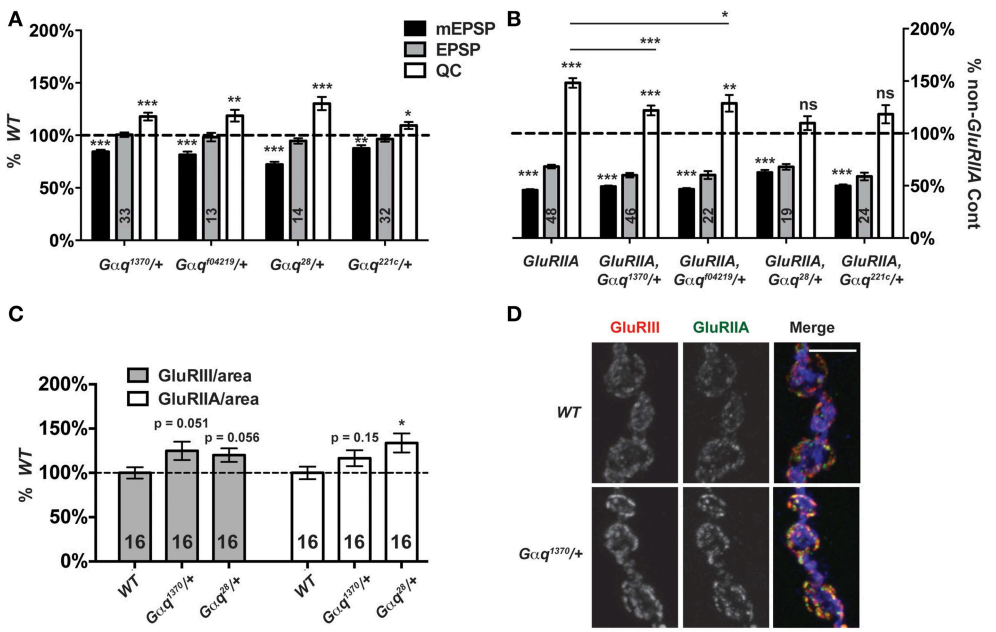

FIGURE 8 | Partial Gα$_q$ loss impairs homeostatic compensation. **(A)** Heterozygous loss of *Gαq* does not affect levels of evoked neurotransmission (ns, $p > 0.05$ for each allele compared to *WT*). However, as with loss of *Plc21C,* there is a small decrease in mEPSP amplitude (**$p < 0.01$ for each allele) and a small increase in QC (p-values vary for each allele, but all < 0.05). **(B)** When challenged with a loss of *GluRIIA*, *Gαq*^{28}/+ and *Gαq*^{221c}/+ NMJs show a complete block in homeostatic compensation (QC—ns, $p > 0.05$) while *Gαq*^{1370}/+ and *Gαq*^{f04219}/+ show partial compensation with quantal content elevated slightly compared to genetic control (***$p < 0.001$ and **$p < 0.01$, respectively, Student's *T*-Test) but not to the full extent seen in the *GluRIIA*^{SP16} control (***$p < 0.001$ and *$p < 0.05$). **(C,D)** NMJ glutamate receptor subunit levels per unit of synapse area are not decreased in *Gαq*/+ mutants; if anything, they may be slightly enhanced (scale bar, 5 μm). Merged images include anti-GluRIII (red), -GluRIIA (green), and -HRP (blue) staining.

Flexibility and Uses of the *T15* Drosophila Stock

The concept of expressing GAL4-responsive *UAS* transgenes both pre- and post-synaptically at the NMJ is not novel, but it is useful. In our study we add the *UAS-GluRIII[RNAi]* transgene to provide a homeostatic challenge to the NMJ. We have utilized *T15* by crossing it with *UAS-RNAi* transgenes and interrogating the synapse electrophysiologically. Variations on this genetic theme beyond RNAi screening are possible. For example, to test whether NMJ overexpression of a particular *UAS*-driven target gene impairs HSP, a single cross to *T15* would be sufficient. In fact, an "overexpression screen" could be conducted by crossing *T15* to Drosophila stocks carrying transposons with *UAS* insertions in orientations that drive gene expression (Rorth, 1996; Beinert et al., 2004; Bellen et al., 2004; Thibault et al., 2004; Staudt et al., 2005). For another example, viable loss-of-function mutations on the X-chromosome could be directly assayed for roles in homeostatic plasticity. This could be done by crossing mutant females to *BG57-GAL4*, *UAS-GluRIII[RNAi]* males and examining the mutant male larval progeny by electrophysiology. Chromosomal deficiency or duplication screens are also feasible by employing the *BG57-GAL4*, *UAS-GluRIII[RNAi]* chromosome in a similar crossing scheme. With *GluRIIA* genetic mutations, such screens or approaches require more extensive genetic work. The single-cross utility of a *BG57-GAL4*, *UAS-GluRIII[RNAi]* chromosome makes large-scale screens to isolate factors involved in developmental or long-term HSP possible. Finally, *T15* does present hurdles. For example, with *T15,* RNAi-targeted genes are impaired both pre- and post-synaptically. Therefore, the screen itself provides limited information regarding tissue specificity of new homeostatic factors. Follow-up experiments examining positives in the pre- and/or post-synaptic compartments need to be done to garner this information. The advantage of being able to identify both pre- and post-synaptic regulators of HSP in a single-generation crossing scheme outweighs the disadvantage of follow-up work.

Ca_V2 Channel Expression Levels and Homeostatic Synaptic Plasticity

Ca_V2 channels help to evoke fast neurotransmission by allowing an influx of calcium into the presynaptic terminal upon neuronal depolarization (for comprehensive reviews on Ca_V channels, see Catterall et al., 2005; Zamponi, 2005). Considerable data from multiple synaptic preparations suggest that Ca_V2 channels also gate neurotransmitter release in a homeostatic fashion (Frank, 2014b). At the Drosophila NMJ, increased Ca_V2 function could be directly responsible for a homeostatic potentiation of presynaptic neurotransmitter release (Frank et al., 2006). Yet it is unclear precisely how this process works. It is not known if increased neurotransmission results from trafficking of new Ca_V2 channels to the synapse, a modulation of functional properties of Ca_V2 channels already present at the synapse—or even a modulation of Ca^{2+}-triggered signaling processes downstream of Ca_V2 function.

Prior data examining *cac* loss-of-function mutants have not answered this question. Hypomorphic cac^S; *GluRIIA* double mutant NMJs have no significant increase in quantal content compared to cac^S mutant NMJs (Frank et al., 2006). Corroborating this finding, presynaptic Ca^{2+} imaging experiments demonstrate that postsynaptic glutamate receptor impairment correlates with an increase of presynaptic calcium transients (Müller and Davis, 2012)—and cac^S mutant NMJs are particularly resistant to potentiation of function after glutamate receptor impairment (Müller and Davis, 2012). A weaker hypomorphic allele, cac^{TS2} can impair homeostatic compensation induced by *GluRIIA* deletion, but only if the cac^{TS2}*; GluRIIA* animals are reared at a high temperature (29°C)—likely lowering effective *cac* gene function (Frank et al., 2006). Together, these data suggest that absolute amount of Cac/Ca_V2 function may correlate with the homeostatic capacity of the synapse. Yet additional observations counter this idea. For instance, cac^S/+ heterozygous NMJs have nearly normal neurotransmission—significantly better than cac^{TS2} homozygous NMJs—but cac^S/+; *GluRIIA* NMJs raised at 22°C have partially impaired synaptic homeostasis, while cac^{TS2}*; GluRIIA* animals reared at 22°C have intact homeostatic compensation (Frank et al., 2006). Additionally, heterozygous null cac^{HC129}/+ animals display normal homeostatic compensation (Frank et al., 2009). Taken altogether, it is possible that the presence of dysfunctional Ca_V2 channel subunits (e.g., Cac^S) throughout development—rather than the absolute degree of Ca_V2 dysfunction—could be the driving factor behind impairments of synaptic homeostasis.

Given these background data, it was important to check if severe diminishment of *cac* function is sufficient to impair homeostatic plasticity at the NMJ. Our analyses of *T15* × *UAS-cac[RNAi]* NMJs demonstrate that synaptic homeostasis is still intact across multiple concentrations of $[Ca^{2+}]_e$, even when there is severely defective baseline neurotransmission (**Figure 5**). It appears if the Ca_V2 channels present at the synapse are wild-type channels, the NMJ retains its homeostatic capacity—even if the number of functional Ca_V2 channels that successfully make it to the terminal is sharply reduced. This result is inconsistent with a model in which Ca^{2+}-directed signaling downstream of Ca_V2 activity drives homeostatic plasticity. However, it does not resolve the question of whether the homeostatic potentiation of release normally proceeds through the addition of functional channels to the synapse or through modulation of channel properties. According to a "slot model" of $Ca_V2.1$ channel positioning, at presynaptic terminals there may exist only a limited number of channel-type-specific slots (Cao et al., 2004). If this were true at the Drosophila NMJ—and if all slots were normally occupied—it would point to modulation of Ca_V2 gating properties as a probable mechanism.

A recent paper corroborates this idea. Mutations in two Drosophila genes encoding epithelial sodium (ENaC) channels block both the rapid induction and long-term expression of synaptic homeostasis—importantly, by impairing necessary increases in presynaptic calcium influx (Younger et al., 2013). Interestingly, when the ENaC antagonist benzamil is acutely applied to *GluRIIA* mutant NMJs, presynaptic calcium influx is dramatically decreased. By contrast, benzamil has no effect on presynaptic calcium influx at wild-type NMJs (Younger et al., 2013). Collectively, these observations are consistent with a model in which Ca_V2 gating properties are enhanced during synaptic homeostasis, with

ENaC channels mediating this enhancement (Younger et al., 2013).

CSP and a Sustained Expression of Homeostatic Plasticity

CSP was originally identified in Drosophila from an antibody-based approach as a synapse-specific antigen (Zinsmaier et al., 1990). Follow-up work has demonstrated that CSP executes multiple synaptic functions (Zinsmaier, 2010; Donnelier and Braun, 2014). Interestingly, loss of CSP function appears to exacerbate synaptic problems over developmental time in organisms as varied as Drosophila (Zinsmaier et al., 1994), mice (Fernandez-Chacon et al., 2004; Garcia-Junco-Clemente et al., 2010), and humans (Benitez et al., 2011; Noskova et al., 2011; Velinov et al., 2012). These facts could be in line with data that CSP is required for the long-term maintenance of homeostatic plasticity—even though CSP is not required for its short-term induction (Dickman and Davis, 2009) (**Figure 6F**).

What is the specific role of CSP during the execution of HSP? This is unclear. Based on the literature, there are several plausible connections. As stated above, modulation of Ca_V2 functional properties could be key to synaptic homeostasis, and CSP has previously been implicated in the modulation of Ca_V2-type calcium channel function (Gundersen and Umbach, 1992). We observe a genetic interaction between *Csp* and *cac* loss-of-function mutations (**Figure 6G**)—however, other studies have failed to find evidence for direct Ca_V regulation by CSP, including studies at Drosophila synapses (Morales et al., 1999; Dawson-Scully et al., 2000). Therefore, other modes of regulation that genetically interact with *cac* are likely. For example, CSP has been implicated in the regulation of Ca^{2+}-triggered exocytosis (Dawson-Scully et al., 2000; Bronk et al., 2001; Dawson-Scully et al., 2007), and recent studies have investigated possible CSP chaperone interactions with SNARE molecules. SNAP25 (t-SNARE) expression is significantly decreased in mice lacking CSPα (Chandra et al., 2005; Zhang et al., 2012). This represents a possible link to homeostatic plasticity because reduction of SNAP25 levels compromises homeostatic signaling at the Drosophila NMJ (Dickman et al., 2012).

Gαq, PLCβ, and Lipid Signals

Our data demonstrate that partial losses of *Gαq* and *Plc21C* gene function partially impair the homeostatic response (**Figures 7**, **8**). The data open several possibilities for presynaptic control of neurotransmitter release and homeostatic plasticity. By one model, diminishment of PLCβ signaling could result in excess PIP_2 at the synapse at the expense of IP_3 and membrane-bound DAG. In other systems, it is well known that DAG activates protein kinase C (PKC), which in turn, activates Ca_V2 channels (Tedford and Zamponi, 2006). In this way, DAG-mediated regulation of Ca_V2 function could be a key determinant in homeostatic plasticity. However, by a second model, it has been reported that *Plc21C* gene function and DAG regulate the abundance of synaptic DUNC-13 at the Drosophila NMJ (Aravamudan and Broadie, 2003). DUNC-13 is a highly conserved protein critical for proper maintenance of SNARE-mediated vesicle exocytosis (Jahn and Fasshauer, 2012; Kasai et al., 2012; James and Martin, 2013), a process previously implicated in homeostatic plasticity at the NMJ (Dickman et al., 2012). There exists evidence that DUNC-13 family molecules could regulate the dynamics of the readily releasable pools (RRP) of synaptic vesicles (Chen et al., 2013). RRP size enhancement is another process implemented during synaptic homeostasis at the NMJ (Weyhersmüller et al., 2011; Müller et al., 2012). For a third model, depressed Gαq/PLCβ signaling could result in lower levels of synaptic IP_3. Canonically, intracellular IP_3 binds to IP_3 receptors and liberates Ca^{2+} from intracellular stores. In turn, Ca^{2+}-dependent signaling cascades could permit the expression of homeostatic plasticity. Finally, PIP_2 itself is known to bimodally regulate Ca_V2 channels: Ca_V2 channels are stabilized by low levels of PIP_2, but inhibited by high levels of PIP_2 (Wu et al., 2002). Interestingly, an amino-acid substitution on the intracellular side of transmembrane domain III S6 on $Ca_V2.1$ alters its affinity for PIP_2. This is the same domain containing the amino-acid substitution in cac^S (Smith et al., 1998; Zhen et al., 2006). More experiments are required to define a precise mechanism of Gαq/PLCβ signaling in the execution of synaptic homeostasis at the NMJ. Regardless of mechanism, the results implicating Gαq and Plc21C in synaptic homeostasis suggest that a synaptic signal controls presynaptic excitability through a G-Protein Coupled Receptor (GPCR)/Gαq/PLCβ-mediated signaling pathway. This is an exciting possibility that is open to exploration via additional genetic approaches in Drosophila, taking advantage of tools like those we generated for this study.

Acknowledgments

We thank many researchers and Drosophila stock centers cited in the Materials and Methods for providing Drosophila stocks. We thank the CAF, T. Tootle, and F. Lin lab members for helpful discussions. We thank Catherine Neff and Thomas James for comments on an earlier version of this manuscript. This work was supported in part by NIH grant NS062738 to CAF, a Whitehall Foundation Grant to CAF, as well as funds from the Department of Anatomy and Cell Biology at the University of Iowa Carver College of Medicine, and a Carver Trust Medical Research Initiative Grant to CAF. AMS was supported in part by National Institutes of Health Predoctoral Training Grant T32GM008629 (PI Daniel Eberl).

Supplementary Material

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fncel.2015.00107/abstract

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in
CELLULAR NEUROSCIENCE

MINI REVIEW ARTICLE
published: 17 June 2014
doi: 10.3389/fncel.2014.00164

Presynaptic mechanisms of neuronal plasticity and their role in epilepsy

Jochen C. Meier[1]*, Marcus Semtner[1], Aline Winkelmann[1] and Jakob Wolfart[2]

[1] *RNA Editing and Hyperexcitability Disorders, Max Delbrück Centre for Molecular Medicine, Neurosciences, Berlin, Germany*
[2] *Oscar Langendorff Institute of Physiology, University of Rostock, Rostock, Germany*

Edited by:
Jaichandar Subramanian, Massachusetts Institute of Technology, USA

Reviewed by:
Detlev Boison, Legacy Research Institute, USA
Rory McQuiston, Virginia Commonwealth University, USA

****Correspondence:***
Jochen C. Meier, RNA Editing and Hyperexcitability Disorders, Max Delbrück Centre for Molecular Medicine, Neurosciences, Building C31.2, Robert-Rössle-Straße 10, 13125 Berlin, Germany
e-mail: jochen.meier@mdc-berlin.de

Synaptic communication requires constant adjustments of pre- and postsynaptic efficacies. In addition to synaptic long term plasticity, the presynaptic machinery underlies homeostatic regulations which prevent out of range transmitter release. In this minireview we will discuss the relevance of selected presynaptic mechanisms to epilepsy including voltage- and ligand-gated ion channels as well as cannabinoid and adenosine receptor signaling.

Keywords: epilepsy, axon, RNA editing, potassium channels, glycine receptor, homeostatic regulation, neuropsychiatric disorders, hippocampus

INTRODUCTION

Many studies highlighted the importance of homeostasis in neuronal signaling either within neurons, then called "intrinsic plasticity" or between neurons, referred to as "synaptic scaling" (Davis and Bezprozvanny, 2001; Eichler and Meier, 2008; Turrigiano, 2011). Classic synaptic plasticity in its non-homeostatic "Hebbian" form and pathological disturbances need counterbalancing homeostatic scaling mechanisms (Abbott and Nelson, 2000). The latter was mainly regarded from the postsynaptic perspective (Thiagarajan et al., 2005; Groth et al., 2011). However, in addition to presynaptic expression of synaptic long term plasticity (Nicoll and Schmitz, 2005), slow homeostatic regulations occur in the presynapse, e.g., in form of chronic receptor or ion channel modulations.

PRESYNAPTIC ION CHANNEL AND GLYCINE RECEPTOR PLASTICITY

Because transmitter release is controlled by action potential-(AP)-triggered calcium influx in the synaptic terminal, regulation of ion channels which shape the axonal AP and terminal depolarization is an effective mechanism of presynaptic plasticity. With this definition, the AP initiation zone (AIZ) could be viewed as part of the presynaptic equipment. A striking form of homeostatic plasticity has been documented for the AIZ: this entire subcellular structure including voltage-gated Na^+ (Na_v) and K^+ (K_v) channels can be shifted along the axon (**Figure 1**), thereby counteracting hyperexcitation by increasing thresholds for AP generation (Grubb and Burrone, 2010). Such axonal remodeling may be facilitated via ion channel trafficking regulated by alternative splicing, as shown for "shaw-related" Kv3 channels (Gu et al., 2012). Other important constituents of presynaptic control are "shaker-related" K_V1 channels (Wang et al., 1994). Their role is well demonstrated for the large glutamatergic mossy fiber boutons of dentate granule cells, which impinge on hippocampal CA3 pyramidal cells (Geiger and Jonas, 2000; Bischofberger et al., 2006). Here, K_v channels could gain further importance during temporal lobe epilepsy (TLE), when seizures invade the hippocampus and feedforward inhibition of CA3 pyramidal cells via interneurons is compromised (Lawrence and McBain, 2003). Indeed, seizures trigger a transcriptional upregulation of $K_v1.1$ channels in granule cells, thereby delaying their AP responses considerably, as recently shown in a TLE mouse model (Kirchheim et al., 2013). Consistent with the view that $K_V1.1$ is a promising antiepileptic target, $K_V1.1$ knockout mice develop epilepsy (Wenzel et al., 2007) and lentiviral overexpression of $K_V1.1$ ameliorates seizures in an animal model of neocortical epilepsy (Wykes et al., 2012). The interaction of activity-dependent downscaling and potentiation of presynaptic excitability may involve the adenylyl cyclase pathway (Nicoll and Schmitz, 2005) but it is still unclear how exactly these seemingly opposed mechanisms interact in the same presynaptic compartment.

Epilepsy often comes with cognitive dysfunction and neuropsychiatric comorbidities (García-Morales et al., 2008). We discovered a molecule which in this regard may have an important impact: an RNA variant of the neurotransmitter receptor for glycine (GlyR). The GlyRs are subject to increased RNA editing in resected hippocampi of TLE patients (Eichler et al., 2008)

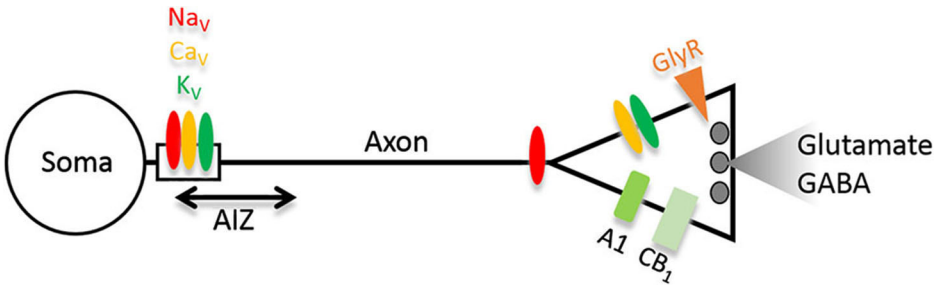

FIGURE 1 | Scheme depicting strategic molecules relevant for homeostasis of presynaptic function and epilepsy discussed in this minireview. In glutamatergic neurons, gain-of-function of molecules colored red/orange will increase network excitability, whereas those colored green will decrease it. The opposite holds true if the changes occur in GABAergic neurons. Abbreviations: Na_V, Ca_V, K_V, voltage-gated sodium, calcium and potassium channels; AIZ, action potential (AP) initiation zone; GlyR, glycine receptors; A1, adenosine receptor; CB_1, cannabinoid receptor 1.

which profoundly influences biophysical receptor properties. The reason is an amino acid substitution in the ligand binding domain leading to gain-of-function receptors with increased neurotransmitter affinity (Meier et al., 2005; Eichler et al., 2008; Legendre et al., 2009) and spontaneous channel activity (Kletke et al., 2013; Winkelmann et al., 2014). In addition, RNA splicing governs presynaptic GlyR expression (Winkelmann et al., 2014), and in hippocampal neurons, the lack of the GlyR β subunit (Weltzien et al., 2012) which governs postsynaptic receptor clustering (Meyer et al., 1995; Meier et al., 2000, 2001; Eichler et al., 2009; Förstera et al., 2010; Kowalczyk et al., 2013), certainly facilitates GlyR expression and function at presynapses (**Figure 1**). Presynaptic GlyRs are tightly packed (∼200 receptor channels in a cluster with ∼100 nm radius; Notelaers et al., 2012, 2014a,b), which implies that a single presynaptic cluster from the spontaneously active GlyR RNA variant will have a considerable functional impact on synaptic neurotransmitter release, even if the contribution of the glycinergic system to this brain region appears limited (Zeilhofer et al., 2005). Consistent with the excitatory nature of presynaptic chloride channels and the well documented presynaptic GlyR expression in the hippocampus (Kubota et al., 2010; Ruiz et al., 2010; Waseem and Fedorovich, 2010; Winkelmann et al., 2014), we found that the spontaneously active GlyR RNA variant actually increased presynaptic excitability and the functional impact of glutamatergic neurons or parvalbumin-positive interneurons *in vivo* and, depending on the type of neuron, triggered cognitive dysfunction or anxiety in our mouse model of epilepsy (Winkelmann et al., 2014). In agreement with the proposed critical role of presynaptic GlyRs in the regulation of neural network excitability, application of a low, non-receptor-saturating, glycine concentration (10 μM) to corticohippocampal slice preparations was sufficient to enhance epileptiform activity induced by block of K_V1 channels (Chen et al., 2014).

RETROGRADE AUTOCRINE AND PARACRINE SIGNALING

Although the idea of cannabis as a potential antiepileptic drug is ancient, it remained elusive how it could work reliably (Adams and Martin, 1996; Miller, 2013). Recent discoveries on endogenous cannabinoid receptors (CB), of which particularly CB_1 is widely expressed in presynaptic terminals of excitatory and inhibitory neurons (**Figure 1**), could lead to a better understanding of CB mechanisms in epilepsy (Alger, 2004; Katona and Freund, 2008; Hill et al., 2012). In GABAergic neurons, activation of CB_1, e.g., via neuronal activity-dependent retrograde post-to-presynapse release of CBs anandamide or 2-AG has been shown to decrease synaptic GABA release, a mechanism termed depolarization-induced suppression of inhibition (DSI; Ohno-Shosaku et al., 2001; Wilson et al., 2001). Consistently, elevated CB_1 presence observed in epilepsy models and TLE patients (Goffin et al., 2011; Karlócai et al., 2011; Bojnik et al., 2012) has been interpreted as proconvulsive (Chen et al., 2003, 2007). On the other hand, CB_1 is also expressed on glutamatergic terminals, where its activation reduces glutamate release (Domenici et al., 2006; Kawamura et al., 2006). Furthermore, CB_1 activation increases inward rectifier K^+ (Kir) currents (Mackie et al., 1995; Chemin et al., 2001) mediated via postsynaptic channels which are also upregulated in TLE (Young et al., 2009; Stegen et al., 2012). In summary, while elevation of CB_1 at GABAergic synapses and reduction at glutamatergic synapses likely constitute endogenous adaptations to epilepsy, exogenous CB_1 overexpression and activation in principal neurons, possibly via receptors physiologically rarely activated, could effectively protect against seizures (Blair et al., 2006; Guggenhuber et al., 2010; Hofmann and Frazier, 2013).

Adenosine triphosphate (ATP) is released from astrocytes and can enhance neuronal excitability through its direct action onto purinergic receptors. However, ATP can also exert indirect effects upon its enzymatic conversion to adenosine and signaling through adenosine A1 receptors, which reduces synaptic glutamate release (Nicoll and Schmitz, 2005; Boison, 2013; Dias et al., 2013). Therefore, adenosine signaling is another mechanism of presynaptic homeostasis with recognized relevance to epilepsy; while too much adenosine clearance via gliosis-enhanced adenosine kinase activity is a proconvulsive factor, adenosine augmentation in the epileptic focus represents a powerful anticonvulsive principle (Boison, 2012).

PERSPECTIVE

In agreement with the proposed critical role of presynaptic compartments in the regulation of neural network homeostasis, diverse pharmacological agents with a presynaptic mode of action were reported to be effective in the treatment of epilepsy. In

particular drugs which provide rapid adaptation against excessive excitation, e.g., via use-dependent inhibition of Na_v or Ca_v channels (phenytoin, carbamazepine, lamotrigine, topiramate, and levetiracetam), likely act primarily in axons (Stefani et al., 1996; Wu et al., 1998; Catterall, 1999; Vogl et al., 2012). Interestingly, these drugs also have effects on psychiatric symptoms (Barbosa et al., 2003; Lexi-Comp, 2009; Andrus and Gilbert, 2010) indicating common underlying mechanisms of cognitive dysfunction and psychiatric symptoms of epilepsy. With more research on neuron type-specific roles in behavior (Lovett-Barron et al., 2014; Winkelmann et al., 2014), new antiepileptic strategies could ground on these insights and specifically target presynaptic molecules in the affected cell types.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 22 April 2014; accepted: 29 May 2014; published online: 17 June 2014.
Citation: Meier JC, Semtner M, Winkelmann A and Wolfart J (2014) Presynaptic mechanisms of neuronal plasticity and their role in epilepsy. Front. Cell. Neurosci. ***8****:164. doi: 10.3389/fncel.2014.00164*
This article was submitted to the journal Frontiers in Cellular Neuroscience.

frontiers in
CELLULAR NEUROSCIENCE

REVIEW ARTICLE
published: 21 November 2013
doi: 10.3389/fncel.2013.00223

Emerging links between homeostatic synaptic plasticity and neurological disease

Joyce Wondolowski and Dion Dickman*

Department of Biology, University of Southern California, Los Angeles, CA, USA

Edited by:
Jaichandar Subramanian, Massachusetts Institute of Technology, USA

Reviewed by:
Michela Matteoli, University of Milano, Italy
Lu Chen, Stanford University School of Medicine, USA
Davide Pozzi, University of Milano (in collaboration with Michela Matteoli), Italy

***Correspondence:**
Dion Dickman, Department of Biology, University of Southern California, 3641 Watt Way, Los Angeles, CA 90089, USA
e-mail: dickman@usc.edu

Homeostatic signaling systems are ubiquitous forms of biological regulation, having been studied for hundreds of years in the context of diverse physiological processes including body temperature and osmotic balance. However, only recently has this concept been brought to the study of excitatory and inhibitory electrical activity that the nervous system uses to establish and maintain stable communication. Synapses are a primary target of neuronal regulation with a variety of studies over the past 15 years demonstrating that these cellular junctions are under bidirectional homeostatic control. Recent work from an array of diverse systems and approaches has revealed exciting new links between homeostatic synaptic plasticity and a variety of seemingly disparate neurological and psychiatric diseases. These include autism spectrum disorders, intellectual disabilities, schizophrenia, and Fragile X Syndrome. Although the molecular mechanisms through which defective homeostatic signaling may lead to disease pathogenesis remain unclear, rapid progress is likely to be made in the coming years using a powerful combination of genetic, imaging, electrophysiological, and next generation sequencing approaches. Importantly, understanding homeostatic synaptic plasticity at a cellular and molecular level may lead to developments in new therapeutic innovations to treat these diseases. In this review we will examine recent studies that demonstrate homeostatic control of postsynaptic protein translation, retrograde signaling, and presynaptic function that may contribute to the etiology of complex neurological and psychiatric diseases.

Keywords: synaptic plasticity, homeostasis, neurological disease, retrograde signaling, presynaptic plasticity

INTRODUCTION

Constraining nervous system activity within stable physiological ranges is critical for robust and reliable brain function. However, this stability must also permit the flexibility necessary for learning and memory to occur during the life experiences of an organism. While various forms of Hebbian plasticity have been shown to potentiate or weaken individual synaptic strengths, these mechanisms are inherently destabilizing and would lead to unconstrained activity if left unchecked. Homeostatic processes have therefore been postulated to counteract the instability generated through Hebbian forces, adjusting synaptic strengths and intrinsic neuronal excitability to keep neural circuits functioning within stable dynamic ranges throughout developmental, experiential, and environmental challenges. While homeostatic plasticity is fundamental and conserved in the nervous systems of invertebrates, mammals, and humans, our understanding of the underlying mechanisms of these complex and robust signaling systems has been quite limited.

In the late 1990s, Gina Turrigiano and colleagues reported robust homeostatic synaptic plasticity in cultured rodent neurons (Turrigiano et al., 1998). Around this same time, investigations in *Drosophila* of postsynaptic receptor mutants at the neuromuscular junction (NMJ) also revealed robust homeostatic control of synaptic strength (Petersen et al., 1997; Davis and Goodman, 1998b). Many groups have since described homeostatic adaptations in diverse systems and organisms. Some of these homeostatic processes are thought to require retrograde signaling processes and presynaptic expression (Davis, 2006) while others appear to be postsynaptically induced and expressed (Turrigiano, 2008; Pozo and Goda, 2010).

Beyond conceptual ideas that disruptions in homeostatic synaptic plasticity could lead, in principle, to neural excitability disorders like epilepsy, compelling links with disease had remained elusive. Although synaptic homeostasis has been demonstrated to be a fundamental signaling system observed in a variety of diverse organisms including crustaceans, *C. elegans*, *Drosophila melanogaster*, rodents, and humans (Grunwald et al., 2004; Turrigiano and Nelson, 2004; Marder and Goaillard, 2006; Pozo and Goda, 2010; Turrigiano, 2012; Vitureira et al., 2012; Frank, 2013), direct associations with disease were unclear. Of course, this was probably due in no small part to our poor understanding, particularly on a molecular and cellular level, of both homeostatic synaptic plasticity and neurological and psychiatric diseases. However, work over the past 5 years has revealed exciting new insights into both of these processes, which in turn has led to the discovery of tantalizing links between diseases of the nervous system and synaptic homeostasis. Although just a beginning, a strong conceptual framework has now been established as a foundation to investigate the extent to which defects in homeostatic synaptic signaling could plausibly contribute to

the disease pathogenesis of an array of neuropsychiatric and neurological conditions.

These intriguing links include disease susceptibility genes on both sides of the synapse that appear to help orchestrate the homeostatic control of synaptic function (**Table 1**) (Pozo and Goda, 2010; Wang et al., 2011a; Yizhar et al., 2011; Qiu et al., 2012). Several theories have been proposed to explain the general mechanisms of neural or synaptic dysfunction that might underlie these disorders (Kehrer et al., 2008; Sudhof, 2008; Yizhar et al., 2011). Disruption or dysregulation of homeostatic synaptic plasticity could be one cause of the excitation/inhibition imbalances that have been recently implicated in cognitive and developmental deficits of the nervous system (Kehrer et al., 2008; Rubenstein, 2010). Indeed, it is tempting to speculate that the high rate of seizures linked with many neurological and neuropsychiatric diseases (Lhatoo and Sander, 2001; Leung and Ring, 2013) could be explained, in part, by defects in homeostatic plasticity. Although compelling studies suggest many of these processes target postsynaptic receptor trafficking and synaptic scaling (**Table 1**), given the topical focus of this FCN issue, this review will focus on the disease-related pathways linked to the retrograde and presynaptic control of homeostatic plasticity (**Figure 1**).

DISEASES ASSOCIATED WITH POSTSYNAPTIC MODULATION OF PROTEIN SYNTHESIS AND HOMEOSTATIC RETROGRADE SIGNALING

Several research groups have revealed a complex homeostatic feedback system in the postsynaptic cell that serves to initiate and convey retrograde signaling information back to the presynaptic neuron. These signaling molecules include retinoic acid (RA) (Wang et al., 2011b), glial-derived TNF-alpha (Stellwagen and Malenka, 2006), endocannabinoids (Zhang et al., 2009; Kim and Alger, 2010), and brain-derived neurotrophic factor (BDNF) (Jakawich et al., 2010; Henry et al., 2012). Both RA and BDNF have been implicated in a variety of biological processes and diseases, and multiple lines of evidence suggest that perturbations to postsynaptic glutamate receptors lead to RA and BDNF synthesis in the postsynaptic cell and subsequent alteration of synaptic protein synthesis and retrograde signaling that homeostatically modulate presynaptic function (Jakawich et al., 2010; Chen et al., 2012; Henry et al., 2012; Kavalali and Monteggia, 2012). Interestingly, in the case of several neuropsychiatric diseases including Tuberous Sclerosis Complex and Fragile X Syndrome, imbalances in postsynaptic protein synthesis have been implicated in their etiology. These neuropsychiatric diseases are associated with intellectual disability and autism spectrum disorders, and may arise due to synaptic pathophysiology because of imbalances in the control of postsynaptic protein synthesis (Auerbach et al., 2011; Martin and Huntsman, 2012). Hence, a core postsynaptic step in homeostatic signaling, control of local protein synthesis, may contribute to retrograde signaling and the synaptic dysfunction contributing to these diseases.

TUBEROUS SCLEROSIS COMPLEX (TSC)

Tuberous Sclerosis Complex is a genetic disease characterized by formation of non-malignant tumors in the brain and other organs (Osborne et al., 1991; Curatolo et al., 2008). This disease is associated with seizures, intellectual disability, and autism, and is the result of heterozygous mutations in the genes encoding *TSC1* or *TSC2*, which encode proteins that together form a complex that regulates activity of the mammalian Target of Rapamycin (mTOR), a well-known regulator of mRNA translation (Zeng et al., 2008; Curatolo and Moavero, 2012; Ryther and Wong, 2012; Talos et al., 2012). mTOR is a constituent of a complex that modulates protein translation by interacting with eIF4E and other translation control factors, suggesting that aberrant regulation of synaptic protein synthesis may contribute to the neurological diseases associated with TSC.

Recent evidence from two established experimental models for homeostatic synaptic plasticity has revealed that a postsynaptic signaling system, which regulates protein expression via the mTOR pathway, is necessary for the retrograde control of presynaptic release. Using rodent hippocampal neurons in culture, researchers have found that disruption of NMDA receptor mediated miniature postsynaptic currents leads to a rapid increase in postsynaptic protein synthesis and a compensatory increase in postsynaptic glutamate receptor expression (Sutton et al., 2006). Further, alterations in synaptic protein synthesis via eIF4E and mTOR were found to be rapidly induced (within 1–2 h) following acute pharmacological blockade of AMPA receptor function using CNQX (Jakawich et al., 2010; Henry et al., 2012). Interestingly, one substrate of this postsynaptic signaling system is BDNF, which is synthesized and released postsynaptically where it activates presynaptic TrkA receptors and enhances presynaptic function (Jakawich et al., 2010). Dysfunction in this acute, retrograde modulation of presynaptic function via postsynaptic protein synthesis could plausibly contribute to the social, cognitive, and excitability disorders observed in TSC and other neurological diseases, including epilepsy.

This same pathway that modulates postsynaptic protein synthesis and leads to the retrograde enhancement of presynaptic transmission via the eIF4E and the TOR translational control factors has also been demonstrated in a separate model system for presynaptic homeostatic plasticity, the *Drosophila* NMJ (Penney et al., 2012). In this system, pharmacological or genetic perturbations to postsynaptic glutamate receptor function, akin to the protocols used in rodent preparations discussed above, lead to a currently unknown retrograde signal the potentiates presynaptic release (Davis and Goodman, 1998b; Davis, 2006; Frank, 2013). Penney and colleagues found that when levels of postsynaptic eIF4E or TOR proteins were reduced, this led to a disruption in the compensatory homeostatic increase in presynaptic release normally observed following reduction of postsynaptic glutamate receptor function (Penney et al., 2012). Although there is no obvious fly counterpart of BDNF and the identity of the retromer is unknown, it appears that fundamental, evolutionarily conserved parallels between Drosophila and rodents in postsynaptic signaling via eIF4E and TOR can modulate the retrograde, homeostatic control of presynaptic release. This fundamental property of synaptic homeostasis may be dysregulated during TSC. Interestingly, expression of another protein, neurexin, is controlled by eIF4E (Gkogkas et al., 2013), and has been linked with autism spectrum disorders (Sudhof, 2008), providing another putative link

Table 1 | Genes and molecules required for homeostatic synaptic plasticity and linked with neurological diseases.

Molecule	Disease	Process	References
Arc/Arg3.1	Angelman Syndrome	Required for homeostatic control of postsynaptic AMPA receptor trafficking in mammalian *in vivo* and *in vitro* studies.	Gao et al., 2010; Beique et al., 2011; Cao et al., 2013; Korb et al., 2013
BDNF	Autism, depression, schizophrenia, neurodegeneration, others.	Demonstrated to act as a retrograde messenger in response to AMPA receptor blockade to induce homeostatic presynaptic potentiation in hippocampal neuronal cultures.	Jakawich et al., 2010
Ca^{2+} Channels	Migraine, ataxia, epilepsy, autism, ADHD, bipolar disorder, depression, and schizophrenia	Required for the expression of presynaptic homeostatic plasticity at the *Drosophila* NMJ and cultured mammalian neurons.	Terwindt et al., 1998; Cao and Tsien, 2005; Frank et al., 2006; Zhao et al., 2011; Smoller et al., 2013
Dysbindin	Schizophrenia	Required presynaptically for the homeostatic increase in synaptic vesicle release at the *Drosophila* NMJ.	Dickman and Davis, 2009; Zuo et al., 2009
FMRP	Fragile X Syndrome, autism, intellectual disability	Necessary for the expression of RA-mediated homeostatic increases in AMPA receptor expression and decreases in GABA receptor expression in rodent hippocampal cultures.	Soden and Chen, 2010; Sarti et al., 2013
MeCP2	Rett Syndrome (autism)	Necessary for cell-autonomous homeostatic synaptic scaling up and increases in cellular excitability in response to reduced circuit activity *in vitro*, sensory deprivation *in vivo* in mouse visual cortex, and for synaptic down-scaling in mouse hippocampal culture.	Blackman et al., 2012; Qiu et al., 2012; Zhong et al., 2012; Sala and Pizzorusso, 2013
mTOR/eIF4E	Tuberous Sclerosis Complex (TCS)	Required postsynaptically for retrograde regulation of presynaptic homeostatic potentiation at the *Drosophila* NMJ and in mammalian neuronal cultures.	Penney et al., 2012; Talos et al., 2012
Acetylcholine receptors (AChRs)	Myasthenia Gravis	Loss of postsynaptic AChRs leads to presynaptic homeostatic compensation in quantal content in human muscle biopsies and mouse models.	Plomp et al., 1992, 1995
Narp	Epilepsy	Narp is necessary for homeostatic increases in interneuron excitatory synapses in response to increased network activity.	Chang et al., 2010
Neurexin Neuroligin	Autism, schizophrenia, Tourette's Syndrome	Important for presynaptic homeostatic increases in quantal content at the mouse NMJ.	Sons et al., 2006; Sudhof, 2008
NMDA Receptors/eEF2	Depression	Blocking NMDA receptors may prevent eEF2 phosphorylation, increasing translation, and possibly leading to postsynaptic up-scaling of AMPA receptors.	Kavalali and Monteggia, 2012
Rab3GAP	Warburg Micro and Martsolf Syndrome	Required presynaptically for the induction and expression of homeostatic increases in release probability at the fly NMJ.	Aligianis et al., 2006; Muller et al., 2011

A summary genes and molecules associated with neurological diseases in a variety of systems that have been implicated in pre- and postsynaptic signaling mechanisms driving the homeostatic control of synaptic strength.

between homeostatic regulation of the synapse and neuropsychiatric disorders.

FRAGILE X SYNDROME (FXS)

Fragile X Syndrome is a neuropsychiatric disorder characterized by intellectual disability, deficits in social interaction, and associated neurological conditions such as seizures (Bagni and Greenough, 2005; Bhakar et al., 2012). This genetic syndrome is the most widespread single-gene cause of autism and inherited form of mental retardation in boys (Hagerman and Hagerman, 2002; Hernandez et al., 2009). FXS is the result of mutations in the fragile X mental retardation gene (*fmr1*), which encodes the Fragile X Mental Retardation Protein (FMRP), and is necessary for normal neural development (Lhatoo et al., 2001b). Research over the past ten years has demonstrated that FMRP is a synaptic RNA binding protein that represses translation of a subset of

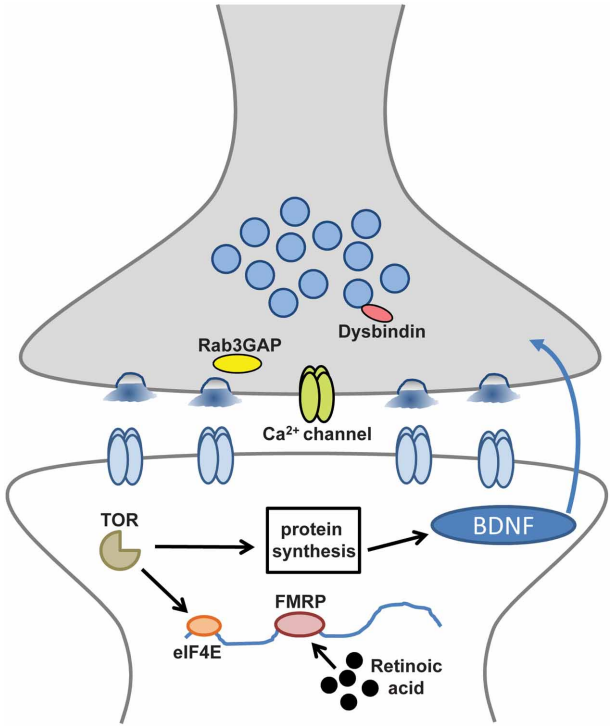

FIGURE 1 | Disease-related molecules and pathways required for retrograde and presynaptic homeostatic signaling. Synaptic components and processes involved in retrograde signaling and presynaptic homeostatic plasticity that may also contribute to neurological and neuropsychiatric diseases.

RNAs; hundreds of FMRP targets have recently been discovered (Bassell and Warren, 2008; Costa-Mattioli et al., 2009; Zukin et al., 2009; Darnell et al., 2011), leading to models in which both pre- and post-synaptic RNAs are under modulatory translational control by FMRP (Feng et al., 1997; Stefani et al., 2004; Christie et al., 2009; Akins et al., 2012).

The intercellular signaling molecule RA has been found to regulate homeostatic synaptic plasticity in rodent neuronal cultures (Aoto et al., 2008; Maghsoodi et al., 2008; Soden and Chen, 2010; Chen et al., 2012). Here, RA was demonstrated to be required for the homeostatic increase in synaptic strength following postsynaptic receptor perturbation (Aoto et al., 2008). Specifically, blockade of excitatory activity was induced by TTX and APV treatment, and RA was necessary for homeostatic postsynaptic scaling through AMPA receptor insertion (Chen et al., 2012). A novel cytosolic and synaptic function for RA was determined, as synthesis of RA was induced following this homeostatic challenge. RA binds to its receptor RARalpha and in turn modulates postsynaptic protein expression (Lane and Bailey, 2005; Chen et al., 2012). Importantly, in addition to the function of RA in regulating postsynaptic homeostatic glutamate receptor scaling, RA was also observed to have roles in modulating presynaptic function, as reflected in increased mEPSC frequency (Wang et al., 2011b; Chen et al., 2012). Intriguingly, FMRP is also necessary for RA-mediated homeostatic plasticity at both excitatory and inhibitory synapses (Soden and Chen, 2010; Sarti et al., 2013). In neurons isolated from *fmr1* mutant mice, bi-directional homeostatic scaling was not observed, while other forms of plasticity remained intact. These findings underscore the importance of tightly regulating synaptic protein synthesis in the context of homeostatic plasticity and the possible neural diseases that may result from dysfunction in this process.

The research described above provides an intriguing body of evidence linking postsynaptic protein synthesis and retrograde signals to both homeostatic synaptic plasticity and neurological diseases. Several diseases show significant overlap in the signaling pathways relevant to trans-synaptic homeostatic signaling. Interestingly, mTOR activity has been implicated in TSC, neurofibromatosis-1, FXS, and a variety of PTEN-associated conditions (Gipson and Johnston, 2012). Regulation of postsynaptic protein synthesis via mTOR and FMRP have both been shown to disrupt mGluR dependent LTD (Auerbach et al., 2011). Indeed, there are even links between FXS and BDNF signaling (Leung and Ring, 2013). The question of how similar signaling systems underlying homeostatic and Hebbian (LTD/LTP) mechanisms are integrated to ensure flexible yet stable synaptic function remains an important area for future research.

Emerging evidence suggests that problems with retrograde signaling may be involved in an array of autism spectrum disorders beyond FXS and TSC. Another example of trans-synaptic signaling being linked to cognitive disease is seen with neurexins and neuroligins, cell-adhesion molecules that are known to regulate synaptic function (Sudhof, 2008). These molecules have been linked with a variety of diseases including autism (Yan et al., 2005; Szatmari et al., 2007; Kim et al., 2008), schizophrenia (Kirov et al., 2008; Walsh et al., 2008), and other neuropsychiatric conditions (Lawson-Yuen et al., 2008; Sudhof, 2008). Intriguingly, neurexins have been found to be necessary for presynaptic homeostatic plasticity at the mammalian NMJ (Sons et al., 2006), providing another possible link between homeostatic plasticity and neuropsychiatric disease.

DISEASES ASSOCIATED WITH PRESYNAPTIC MECHANISMS OF HOMEOSTATIC PLASTICITY

CALCIUM CHANNEL AND HOMEOSTATIC SIGNALING IN MIGRAINE AND EPILEPTIC DISEASES

Migraine is the most common neurological disease, thought to affect 10–15% of the general population (Goadsby et al., 2002; Wessman et al., 2007). Insight into the etiology of these often debilitating headaches may be uncovered through the study of familial hemiplegic migraine type 1 (FHM1), a rare, inherited form of the disease (Ophoff et al., 1996). FHM1 is caused by missense mutations in P/Q type calcium channels, which are involved in neurotransmitter release and presynaptic function (Zhang et al., 1993; Lhatoo et al., 2001a). These mutations cause impairments in the ability of these channels to conduct Ca^{2+} currents triggered by action potentials (Kraus et al., 1998; Hans et al., 1999; Cao et al., 2004). P/Q-type calcium channels have fundamental roles in mediating fast synaptic transmission at nerve terminals. Autosomal dominant mutations in the CACNA1A gene, which encodes the voltage-gated P/Q-type calcium channel alpha1 subunit, has also been associated with cerebellar ataxia, vertigo,

epilepsy, and schizophrenia (see below) in addition to FHM (Lhatoo et al., 2001a). CACNA1 and CACNB2 channelopathies have also been implicated in several other diseases, including autism, attention-deficit hyperactivity disorder, bipolar disorder, and major depressive disorder, indicating that calcium channel signaling may have crucial functions that, when perturbed, may contribute to psychopathological susceptibility (Terwindt et al., 1998; Lee et al., 2013; Smoller et al., 2013). These studies also suggest a disruption in the balance of excitatory and inhibitory activity that may ultimately lead to the pathogenic states of these diseases.

Interestingly, mutations in the *Drosophila* P/Q calcium channel *cacophony* (*cac*) have been found to disrupt homeostatic plasticity at the fly NMJ (Frank et al., 2006, 2009). Hypomorphic mutations in *cac* lead to reduced presynaptic calcium influx and prevent the retrograde, homeostatic increase in presynaptic release. Recent studies using calcium imaging techniques demonstrate that an acute increase in presynaptic calcium influx through Cac is necessary for the expression of homeostatic plasticity (Muller and Davis, 2012; Younger et al., 2013). In addition, regulation of presynaptic calcium influx is also required for homeostatic synaptic plasticity in the mammalian hippocampus (Zhao et al., 2011). Since calcium signaling is known to play a critical role in many aspects of neurotransmission, the function of these channels in neurological disease is certainly not limited to homeostatic plasticity. This topic is covered in more detail in a forthcoming review (Frank, *in review*).

MYASTHENIA GRAVIS (MG)

Myasthenia gravis is an autoimmune disease that causes muscle weakness and fatigue. In a majority of cases, a loss of postsynaptic acetylcholine (ACh) receptors is observed at the NMJ due to autoantibody production targeting these receptors. In recordings from muscles obtained from MG patients and also from toxin-induced animal models of the disease, miniature end-plate potentials (mEPPs) show dramatically reduced amplitudes (Plomp et al., 1992, 1995). However, evoked end-plate potentials (EPPs) are not significantly reduced due to increased quantal content, indicating that homeostatic processes at the human NMJ, like that of the fly NMJ, compensate for this perturbation of postsynaptic receptor function (Cull-Candy et al., 1980; Plomp et al., 1992, 1995; Davis and Goodman, 1998a). This homeostatic increase in ACh release could compensate for the reduced postsynaptic excitability of the muscle. However, if the compensation is not sufficient to restore all EPPs to suprathreshold activation, then defects in muscle activity would still be observed. Indeed, in a subset of recordings mEPP amplitudes were below the detection limit and showed smaller than normal EPPs, likely indicating limitations in homeostatic compensation at these synapses (Plomp et al., 1995). Furthermore, the increased release probability likely contributes to increased short-term depression, leading to rapid exhaustion of neurotransmitter vesicles and rapid-onset muscle fatigue. Ultimately, disruption of postsynaptic receptor function supersedes the homeostatic processes and major disease progression ensues.

A subclass of MG is caused by production of antibodies that attack muscle specific kinase (MuSK) (Hoch et al., 2001; Viegas et al., 2012), a postsynaptic receptor tyrosine kinase known to be important in receptor clustering during NMJ synaptic development and maintenance (DeChiara et al., 1996; Sanes and Lichtman, 2001; Kong et al., 2004). In recordings from muscles obtained from MuSK MG patients (Niks et al., 2010) and from mice immunized against MuSK (Viegas et al., 2012), mEPPs were found to be significantly reduced. However, in contrast to the NMJ studies in mice and human discussed above, there was no evidence of increased presynaptic release in MuSK-dependent MG, indicating a lack of homeostatic expression (Niks et al., 2010; Viegas et al., 2012). Compared to AChR-associated MG, MuSK-related MG is more severe, is treatment-resistant, and is observed more broadly in the musculature, affecting bulbar, facial, and respiratory muscles as well (Evoli et al., 2003), which is likely due, in part, to a lack of homeostatic compensation. These experiments suggest that MuSK may be required as part of the postsynaptic homeostatic signaling system that initiates a retrograde, presynaptic change in release.

WARBURG MICRO AND MARTSOLF SYNDROME

Rab3 proteins are known to regulate vesicular membrane transport and play a key role in modulating calcium-dependent neurotransmitter release (Sakane et al., 2008). The activity of Rab3 is regulated in part by Rab3 GTPase-activating protein (Rab3GAP), which converts active Rab3-GTP to in the inactive GDP-bound form. Rab3GAP is a heterodimeric protein composed of catalytic and non-catalytic subunits, mutations of which cause Warburg Micro syndrome and Martsolf syndrome (Aligianis et al., 2006). Warburg Micro syndrome is an autosomal recessive disorder that causes severe intellectual disability, ocular defects, and microcephaly (Dursun et al., 2012). Martsolf syndrome shares many clinical features with Warburg Micro syndrome, but is less severe and is associated with milder cognitive dysfunction (Aligianis et al., 2006). Recent evidence suggests that Rab3GAP is critically involved in the induction and long-term maintenance of homeostatic synaptic plasticity. Drosophila Rab3GAP mutants do not show homeostatic increases in neurotransmitter release at the NMJ in response to pharmacological or genetic disruption of postsynaptic glutamate receptors (Muller et al., 2011). Rab3GAP appears to work through Rab3 GTPase to negatively regulate the expression of presynaptic homeostatic plasticity. Thus, the disruption of homeostatic synaptic plasticity may contribute to the cognitive and developmental defects associated with these Warburg Micro and Martsolf syndromes.

SCHIZOPHRENIA

Calcium signaling, through the implication of CACNA1, has been implicated in several brain disorders including schizophrenia, and a fly calcium channel, Cac, has been found to block homeostatic synaptic plasticity (see above). In addition to these findings, genetic screens in *Drosophila* have revealed unanticipated links with schizophrenia. As alluded to above, the *Drosophila* NMJ has been established as a model system to characterize homeostatic synaptic plasticity. Pharmacological or genetic manipulations that reduce postsynaptic receptor function lead to a compensatory increase in presynaptic release that precisely offsets the perturbation and restores normal postsynaptic excitability (Davis and

Goodman, 1998b; Davis, 2006; Frank, 2013). Given the rich genetic resources available in flies, electrophysiology-based forward genetic screens have recently been pioneered to identify genes, that when mutated, lead to defective homeostatic synaptic plasticity (Dickman and Davis, 2009; Muller et al., 2011).

One of the first genes identified from these screening efforts was the *Drosophila* homolog of the vertebrate gene *dysbindin* (Dickman and Davis, 2009). Interestingly, the human homolog of this gene, *DTNBP1*, has been identified as a primary schizophrenia susceptibility gene in a variety of Genome Wide Association Studies (Ross et al., 2006; Zuo et al., 2009; Ghiani and Dell'Angelica, 2011). In Drosophila, Dysbindin was found to be required presynaptically for both the acute induction and long term expression of synaptic homeostasis (Dickman and Davis, 2009). Dysbindin localized to presynaptic vesicles and was demonstrated to alter the calcium dependence of synaptic vesicle release in a unique way, where mutants exhibited severely reduced baseline release, but only at low external calcium concentrations, whereas overexpression of *dysbindin* led to a potentiation of baseline release, but only at higher external calcium levels (Dickman and Davis, 2009). Subsequent work found that Snapin, a synaptic vesicle protein that binds to Dysbindin, was also required for homeostatic synaptic plasticity and was necessary for the potentiation of baseline synaptic transmission by Dysbindin (Dickman et al., 2012). In rodent studies, *dysbindin* was found to be necessary for proper synaptic glutamate release and neurotransmission (Numakawa et al., 2004; Chen et al., 2008), while mouse mutants even displayed behavioral deficits akin to schizophrenia-like conditions (Chen et al., 2008). It is thus interesting to speculate that Dysbindin may homeostatically tune synaptic strength, and deficits in this process may give rise to cognitive and behavioral conditions of relevance to the etiology of schizophrenia.

Dysbindin and Snapin are part of a larger protein complex called the Biogenesis of Lysosome-related Organelles Complex-1 (BLOC-1), composed of eight total subunits (Ghiani and Dell'Angelica, 2011; Mullin et al., 2011). While the molecular function of the BLOC-1 complex is unclear, it appears to be involved in vesicle trafficking (Ghiani and Dell'Angelica, 2011; Larimore et al., 2011). Consistent with such a role in the nervous system, both Dysbindin and Snapin appear to function in synaptic vesicle trafficking and may be involved in other synaptic membrane trafficking pathways. Interestingly, another component of the BLOC-1 complex, Muted, has recently been associated with schizophrenia (Morris et al., 2008; Ryder and Faundez, 2009). These findings underscore the intriguing possibility that defective synaptic membrane trafficking through components of the BLOC-1 complex in the context of homeostatic synaptic plasticity may contribute to the etiology of complex neuropsychiatric diseases like schizophrenia.

CONCLUDING REMARKS

Tantalizing links have recently been uncovered that suggest synaptic processes necessary for the homeostatic control of synaptic function, when defective, may contribute to the pathogenesis of a variety of neurological and neuropsychiatric diseases. Importantly, several intriguing and novel genes and molecules have been revealed to disrupt these complex and adaptive homeostatic signaling systems (**Figure 1**). The challenge moving forward will be to understand how these individual genes and molecules work together to integrate homeostatic information and orchestrate stable cellular, synaptic, circuit, and systemic functions in the brain. It is tempting to speculate that the etiologies of many seemingly disparate neurological and neuropsychiatric diseases share common dysfunctions in pathways related to homeostatic synaptic plasticity. Indeed, a large genome-wide study sponsored by the National Institute of Mental Health has revealed a high degree of overlap in the genes responsible for five of the most common neuropsychiatric diseases (Lee et al., 2013). A combination of human genetic mapping and patient studies coupled with innovative genetic, electrophysiological, and imaging approaches in model systems are certain to reveal new insights into the processes driving the homeostatic control of synaptic function in health and disease.

ACKNOWLEDGMENTS

This work was supported by an award from the Ellison Medical Foundation and a grant from the National Institute of Mental Health (MH092351) to Dion Dickman.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 25 August 2013; accepted: 03 November 2013; published online: 21 November 2013.

*Citation: Wondolowski J and Dickman D (2013) Emerging links between homeostatic synaptic plasticity and neurological disease. Front. Cell. Neurosci. **7**:223. doi: 10.3389/fncel.2013.00223*

This article was submitted to the journal Frontiers in Cellular Neuroscience.