HOMEOSTATIC SYNAPTIC PLASTICITY: FROM SYNAPTIC CIRCUIT ASSEMBLY TO NEUROLOGICAL DISORDERS

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HOMEOSTATIC SYNAPTIC PLASTICITY: FROM SYNAPTIC CIRCUIT ASSEMBLY TO NEUROLOGICAL DISORDERS

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EDITORIAL
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Editorial: Homeostatic Synaptic Plasticity: From Synaptic Circuit Assembly to Neurological Disorders

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Editorial on the Research Topic

Homeostatic Synaptic Plasticity: From Synaptic Circuit Assembly to Neurological Disorders

Neuronal networks can be viewed as learning and memory storage devices. They are highly "plastic," changing the way they process information in response to external stimuli. Yet, they are also highly "tenacious," with many neuronal networks retaining their functional identity over many years. Hebbian forms of synaptic plasticity, such as long-term potentiation (LTP) and long-term depression (LTD), use positive feedback mechanisms to either reinforce the more active synapses or weaken those that are less active, thus contributing to neuronal networks tuning their outputs to ever-changing external stimuli. By contrast, homeostatic forms of plasticity use negative feedback mechanisms to maintain the overall neuronal output as close as possible to an "internal" prefixed set point, thus restraining neuronal networks from becoming either silent or hyper-excitable. Recent findings have clearly shown that there is not one but multiple forms of homeostatic plasticity occurring at different levels of organization of the brain, from single synapses to dendritic branches to individual neurons to full neuronal networks (Davis, 2013; Nelson and Valakh, 2015; Mullins et al., 2016).

This ebook presents a collection of articles covering molecular and cellular mechanisms that drive forms of homeostatic plasticity whose dysfunction has been proposed to underlie the pathophysiology of many neurological disorders, such as autism spectrum disorder (ASD), schizophrenia, Alzheimer's disease, addiction, intellectual disability, depression and epilepsy (Wondolowski and Dickman, 2013; Fernandes and Carvalho, 2016; Jaudon et al., 2020; Kavalali and Monteggia, 2020). In particular, the Research Topic explores two possibilities to interpret the diseased brain in light of homeostatic plasticity mechanisms. First, neurological disorders could arise because homeostatic plasticity fails to compensate for genetic defects. This can occur either when the genetic mutation directly impairs built-in feedback control systems or when it is so disruptive to overwhelm the buffering capacity of homeostatic plasticity. Second, as it is often the case for epilepsy, ASD or addiction, homeostatic plasticity can become maladaptive. This can occur when deficits at one level of organization of the nervous system (for example impaired synaptic transmission) are compensated for at a different level of organization (for example by heightened cell-wide intrinsic excitability). While such homeostatic compensations can effectively preserve the overall output of a neuronal network, they are also likely to make it unstable or modify how it processes information.

Homeostatic compensations are often presented as relatively slow processes developing in

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response to prolonged perturbations of neuronal activity and relying on the synthesis of new proteins that regulate key physiological parameters, such as synaptic efficacy, synapse number and membrane excitability. At the transcriptional level, the RE-1 Silencing Transcription factor (REST1) is ideally suited to achieve homeostatic plasticity as it has been shown to repress the expression of various channels and synaptic proteins and has been linked to both homeostatic plasticity and epilepsy. However, the actual role of REST in epilepsy, whether protective or pro-epileptogenic is debated. To clarify the role of REST in epileptogenesis, Carminati et al. have developed a genetic competitive inhibitor to modulate REST activity *in vivo*. The authors demonstrate that inhibiting REST1 reduces the susceptibility to kainate-induced seizures and correlates with an increased expression of REST1 target genes, including potassium channels, GABAergic and glutamatergic receptors. In their perspective article, Lignani et al. further discuss the complex and dynamic functions of REST as well as of one of its targets, HCN1, to better understand the homeostatic adaptations that take place in epilepsy, and why they invariably fail to suppress seizures. The authors propose that a chronic dysregulation of gene expression (the "genetic load") could transform the contribution of REST and HCN1 from homeostatic to pro-epileptogenic. Accordingly, external genetic interventions (e.g., by enhancing the expression of the potassium channel Kv1.1) may push back neural networks within their physiological boundaries and allow them to take back control of their own homeostasis. Maladaptive homeostatic response is also reported by Yeates and Frank at the *Drosophila* neuromuscular junction (NMJ), where impairment of intracellular calcium gates leads to excessive homeostatic presynaptic depression in response to chronic upregulation of the vesicular glutamate transporter (VGLUT).

Downstream of transcription, Thalhammer et al. discuss the emerging role of activity-dependent alternative splicing as a versatile mechanism to optimize homeostasis. This process not only expands the diversity of isoforms encoded by a single gene but also affects the spatiotemporal dynamics of the corresponding transcripts. The authors provide examples of genes which undergo activity-dependent alternative splicing and whose splice variants exhibit divergent -sometimes opposite- functions in compensating for activity perturbations. Those genes include REST1, the scaffolding protein Homer1 and the P/Q type calcium channels, which regulate intrinsic plasticity, synaptic scaling and presynaptic homeostasis, respectively. More recently, also alternative splicing of BK channels has been shown to participate to homeostatic adaptations by contributing to action potential widening in response to network inactivity (Li et al., 2020). Further downstream along the line of gene expression, protein translation is actively regulated to control homeostatic plasticity in time and space. Dubes et al. review the recent literature addressing the role of microRNAs in various forms of homeostatic plasticity. These non-coding RNAs control the translation of multiple homeostatic effectors including channels, receptors, RNA-binding proteins and cytoskeleton-related proteins. The authors highlight the ability of microRNAs to control homeostasis by repressing their targets either cell-wide or in a compartmentalized fashion (i.e., remotely from the cell body), thus providing autonomy to subcellular functional units such as synapses and dendritic branches.

Whether cell-wide or local, homeostatic plasticity ideally should not compromise information processing occurring at various types of synaptic inputs and outputs. Indeed, most neurons receive synaptic inputs from multiple sources while projecting their axon onto distinct targets, where they form synapses displaying specific functional features. In their study, Goel et al. use the *Drosophila* NMJ to investigate how synapses from an individual neuron homeostatically adapt their strength according to the muscle targets they innervate. The authors identify target-specific homeostatic mechanisms that simultaneously balance for hypo- and hyper-innervation through a differential contribution of pre- and post-synaptic signaling pathways. This study thus highlights the diversity of the homeostatic mechanisms simultaneously implemented by a single neuron to accommodate the requirements of multiple types of outputs. In line with these findings, Lee and Kirkwood review recent evidence showing that neurons embedded in complex sensory networks of the mammalian CNS implement homeostatic synaptic plasticity in an input-specific manner following sensory deprivation. They discuss the role of the "sliding threshold" as a major *in vivo* mechanism to homeostatically adjust the propensity for future LTP and LTD at individual connections depending on prior experience. In contrast, synaptic scaling, in which the efficacy of all synapses is uniformly modified, may occur to stabilize neuronal activity under more extreme activity perturbations, for instance following pharmacological manipulations or widespread seizures.

Whether homeostatic adaptations also take place in more physiological situations (e.g., when a subset of synapses undergo Hebbian plasticity) is an enduring question in the field. While Hebbian plasticity is rapidly implemented, homeostatic plasticity is often viewed as a slow process. Yet, both types of plasticity share common signaling pathways and it remains unclear how homeostatic plasticity can operate without erasing Hebbian plasticity. Galanis and Vlachos propose that Hebbian and homeostatic plasticities coexist at the same synapses, thereby limiting each other. In their model, Hebbian plasticity corresponds to the readjustment of the homeostatic set-point allowing for long-term changes to occur at recruited synapses. In turn, the failure of Hebbian plasticity observed in some physiological or pathological situations may represent enhanced homeostasis. The authors further propose a role for the proteolytic processing of the amyloid precursor protein to set the balance between homeostatic and Hebbian synaptic plasticity. Kruijssen and Wierenga discuss an alternative hypothesis, namely that homeostatic plasticity, rather than affecting directly synaptic strength, modifies the ability of synapses to undergo future LTP, depending not only on their own prior experience (the "sliding threshold" hypothesis discussed by Lee and Kirkwood) but also on that of the nearby synapses. In turn, eliciting LTP at individual synapses triggers compensatory changes at nearby synapses through heterosynaptic signaling. The idea that distinct inputs converging onto the same neuron can balance each other is also proposed by Bannon et al. as a mechanism to prevent the runaway dynamics

inherent to Hebbian plasticity. In their review, the authors highlight the possible role of weight-dependent heterosynaptic plasticity in normalizing the excitatory drive to hippocampal inhibitory neurons.

Besides synapse-specific mechanisms, mounting evidence point to both permissive and instructive roles of the extracellular matrix (ECM) and glial cells in homeostatic plasticity. Cingolani et al. discuss how ECM remodeling controls localization and function of various types of metabotropic receptors (for glutamate, dopamine, and serotonin). In turn, metabotropic signaling modulates the extracellular environment, for example, by stimulating extracellular proteases. This synergistic crosstalk stabilizes network activity by regulating both synaptic and intrinsic forms of homeostatic plasticity. In a similar vein, Heir and Stellwagen review how the pro-inflammatory cytokine Tumor necrosis factor alpha (TNFα), which is mainly secreted by glial cells, controls various forms of homeostatic plasticity both *in vitro* and *in vivo* by modulating receptor trafficking. Importantly, both ECM and glial factors are amenable to therapeutic interventions, for example for the control of epileptogenesis (Korotchenko et al., 2014).

Finally, the systematic review by Moulin et al. reports some of the strengths and pitfalls of the research carried out in the field of homeostatic plasticity, focusing on the synaptic scaling literature. In addition to the lack of transparency and details regarding experimental and analysis procedures in some research articles, the authors highlight the underrepresentation of studies using *in vivo* models as well as of those investigating functional interactions with Hebbian plasticity. Like the authors, we believe that such studies should be encouraged in the future.

In summary, this Research Topic provides an overview of recent advances in the field of homeostatic plasticity highlighting the complexity and dynamics of the molecular and cellular mechanisms involved. Perhaps more importantly, most articles presented here not only link homeostatic plasticity to neurological diseases such as epilepsy, neurodegenerative and neuropsychiatric disorders but also provide insights into new avenues for therapeutic intervention.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Single Synapse LTP: A Matter of Context?

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The most commonly studied form of synaptic plasticity is long-term potentiation (LTP). Over the last 15 years, it has been possible to induce structural and functional LTP in dendritic spines using two-photon glutamate uncaging, allowing for studying the signaling mechanisms of LTP with single synapse resolution. In this review, we compare different stimulation methods to induce single synapse LTP and discuss how LTP is expressed. We summarize the underlying signaling mechanisms that have been studied with high spatiotemporal resolution. Finally, we discuss how LTP in a single synapse can be affected by excitatory and inhibitory synapses nearby. We argue that single synapse LTP is highly dependent on context: the choice of induction method, the history of the dendritic spine and the dendritic vicinity crucially affect signaling pathways and expression of single synapse LTP.

Keywords: synaptic plasticity, long-term potentiation, dendritic spine, glutamate uncaging, molecular pathways, synaptic crosstalk

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INTRODUCTION

Synaptic plasticity is the fundamental cellular correlate of learning. By the strengthening and weakening of specific connections, information processing in the brain is changed and memories are formed. The most studied form of plasticity is long-term potentiation (LTP). As first identified in the rabbit brain by Bliss and Lømo (1973), repeatedly stimulating synapses can lead to long lasting enhancement of synaptic strength. This phenomenon has been extensively studied and characterized in a variety of brain regions and species. The majority of studies use electrical stimulation of axon bundles to induce and measure LTP in brain slices. LTP can also be induced pharmacologically by applying for example an N-methyl-d-aspartate (NMDA) receptor agonist. These approaches induce LTP in bulk: many synapses on dendritic branches of multiple neurons are potentiated at the same time. Electrophysiological recordings and biochemical analysis of the underlying signaling pathways have provided significant insights into the mechanisms of LTP (Malenka and Bear, 2004; Citri and Malenka, 2008; Sjöström et al., 2008; Mayford et al., 2012; Bliss and Collingridge, 2013; Herring and Nicoll, 2016; Nicoll, 2017; Diering and Huganir, 2018). However, this way of inducing LTP does not reflect the physiological situation very well. Under physiological conditions, synaptic inputs are usually not synchronously active in such large numbers, and synaptic plasticity presumably takes place at the scale of individual or small groups of synapses.

The development of two-photon glutamate uncaging almost 20 years ago (Matsuzaki et al., 2001, 2004; Ellis-Davies, 2019) made it possible to activate and potentiate individual synapses. Using a caged compound of the main excitatory neurotransmitter, individual excitatory synapses on spines can be activated with focused laser light at a near-physiological spatial and temporal scale (Matsuzaki et al., 2001) and plasticity can be induced by repetitive stimulation (Matsuzaki et al., 2004). Since then, many studies have used two-photon glutamate uncaging to study the induction,

expression and signaling pathways of LTP in single synapses. These studies have significantly improved our understanding of the mechanisms underlying LTP at the single synapse level. However, differences and disagreements between studies also reveal the limitations of our current understanding of single synapse LTP.

The goal of this review is to summarize and compare studies that used two-photon glutamate uncaging to gain insight into single synapse LTP signaling pathways. We will compare different methods to induce LTP in single synapses and discuss how the choice of LTP induction protocol may affect LTP expression and signaling pathways. We will summarize the signaling pathways that are triggered in a single spine during LTP induction using two-photon uncaging and discuss the possibility that multiple LTP pathways may exist, which can be differentially activated depending on the experimental conditions. Finally, we discuss how LTP at a single synapse can affect plasticity at other excitatory and inhibitory synapses on the same dendrite, suggesting that potentiation of an individual synapse should always be considered in the context of its direct dendritic vicinity.

INDUCTION OF SINGLE SYNAPSE LTP

Two-photon microscopy (Denk et al., 1990; Masters and So, 2004) utilizes the physical principle of two-photon excitation: fluorescent proteins are excited only in a femtoliter-sized volume inside the laser beam focus, where the laser light intensity is high enough for excitation by two coincident photons (Zipfel et al., 2003; Svoboda and Yasuda, 2006). Individual long wavelength photons have low energy, which means that out-of-focus laser light causes minimal photodamage. In addition, long wavelength light can penetrate deep into tissue without scattering, allowing live two-photon imaging of small structures, such as dendritic spines up to 1 mm deep into living brain tissue, to be performed (Denk and Svoboda, 1997; Helmchen and Denk, 2005). With the same precision, the two-photon principle allows for precise photolysis of "caged compounds" – biologically active molecules that are inert until exposed to the right wavelength of light (Soeller and Cannell, 1999). The development of MNI-glutamate, a caged compound of the main excitatory neurotransmitter which has a high two-photon cross section, allowed stimulation of single excitatory synapses (Matsuzaki et al., 2001) and induction of plasticity at individual spines (Matsuzaki et al., 2004). The development of several Förster Resonance Energy Transfer (FRET) probes that can detect the activity of signaling molecules on the level of the single spine allowed studying the underlying pathways of LTP with greater detail than ever before (Yasuda, 2012; Ueda et al., 2013; Nakahata and Yasuda, 2018). With these technological advancements, it is now possible to elucidate the mechanisms that are involved in LTP on the level of single excitatory synapses.

The first study to report single synapse LTP was performed by Matsuzaki et al. (2004). Upon performing repeated glutamate uncaging on single dendritic spines, the stimulated spines rapidly grew and remained enlarged for up to 100 min, while unstimulated spines on the same dendrites were unaffected. The authors furthermore showed that spine growth crucially depended on NMDA receptor activation and was similar to spine growth after electrical stimulation. Spine growth was accompanied by a corresponding increase in A-Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic Acid (AMPA) receptor-mediated postsynaptic currents, linking growth of the spine head with functional plasticity of the excitatory synapse. Since the pioneering work by Matsuzaki and colleagues, the two-photon glutamate uncaging technique was quickly adopted by the LTP field, and multiple labs have performed single synapse LTP experiments since then. A major benefit of using glutamate uncaging to study LTP is the high spatial and temporal precision of the stimulus. As presynaptic stimulation is no longer required, it allows for isolating the postsynaptic component of LTP.

Single synapse LTP is generally induced by repeated uncaging pulses. The repeated activation of postsynaptic glutamate receptors results in calcium influx, most prominently via NMDA receptors, which triggers plasticity at the stimulated spine. Induction protocols for LTP differ in several aspects, which may significantly influence downstream signaling and LTP expression. The number of uncaging pulses typically ranges from 30 to 60, and the stimulation frequency usually lies between 0.5 and 2 Hz. Both these parameters will likely affect the total amount of calcium entering the postsynaptic cell and the level of activation of downstream calcium sensing proteins (Fujii et al., 2013). The duration of a single uncaging pulse typically lies between 0.5 and 6 ms. The pulse duration determines the time receptors are exposed to glutamate as well as the total amount of glutamate that is uncaged, affecting the duration and level of activation of glutamate receptors (AMPA receptors and NMDA receptors) in the postsynapse. The uncaging beam is typically aimed 0.5 μm from the spine head to prevent photodamage to the spine. The distance between the location of glutamate release and the spine will impact the diffusion time of glutamate to the receptors. While glutamate uncaging is highly local, especially during strong stimulation glutamate spillover to extrasynaptic receptors and presynaptic receptors (such as metabotropic glutamate receptors) is likely to occur (Rusakov and Kullmann, 1998; Chalifoux and Carter, 2011).

NMDA receptor activation is one of the crucial events for LTP to occur, and different methods are used to ensure NMDA receptor activation during glutamate uncaging at spines (**Figure 1**). Here, we roughly divide these protocols into two categories. The first category is based on the protocol by Matsuzaki and colleagues. To achieve NMDA receptor activation, glutamate uncaging is performed in absence of extracellular magnesium ions to remove blockage of the channel pore (Tanaka et al., 2008; Lee et al., 2009; Patterson et al., 2010; Tønnesen et al., 2014; Oh et al., 2015; Harward et al., 2016). Caged compounds are known to exhibit antagonist activity at gamma-aminobutyric acid $(GABA)_A$ receptors (Fino et al., 2009; Matsuzaki et al., 2010; Ellis-Davies, 2019). Therefore, tetrodotoxin (TTX, a sodium channel blocker) is usually added to the bath solution under magnesium-free conditions to prevent epileptiform-like activity and unwanted plasticity. The second category of protocols pairs glutamate uncaging with postsynaptic depolarization or postsynaptic action potentials to relieve the magnesium block from the NMDA receptors. This type of protocol typically requires electrical access to the postsynaptic cell via a patch clamp

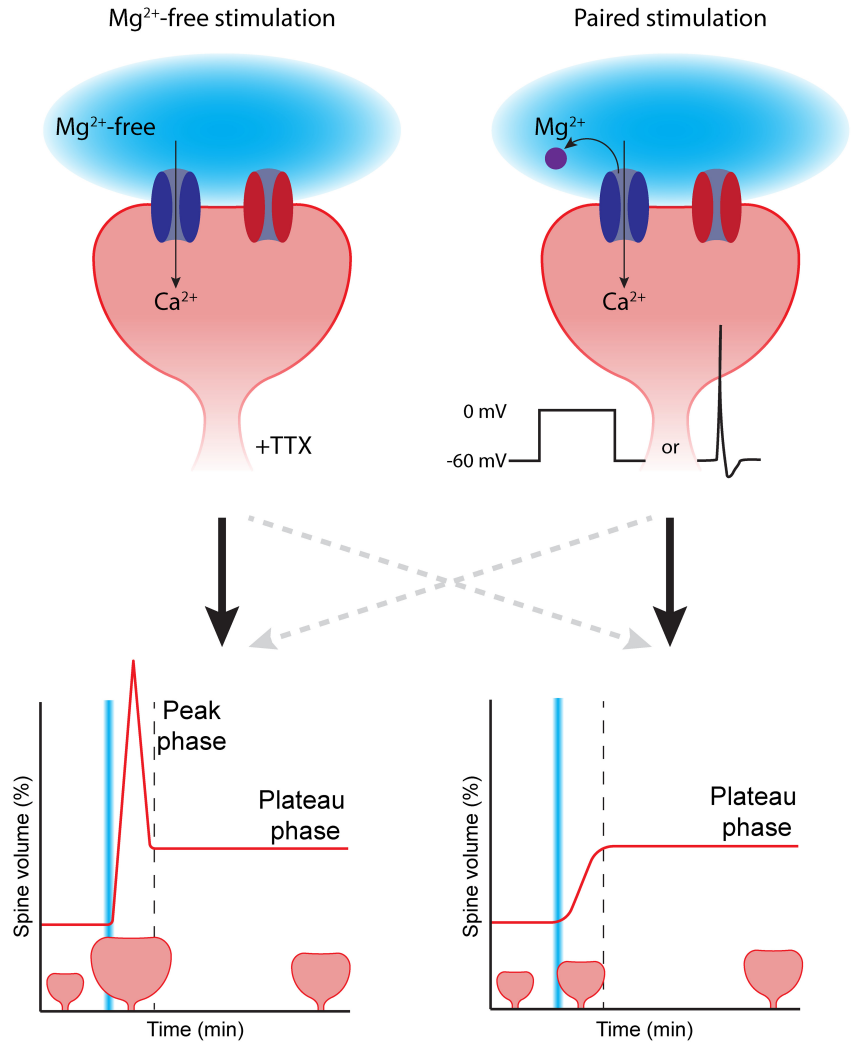

FIGURE 1 | The choice of LTP induction method can affect spine growth. To induce spine growth and functional LTP in single synapses, activation of NMDA receptors (dark blue) is required. Removing the magnesium block (purple) from the NMDA channel pore can be achieved in two ways: **(Left)** glutamate uncaging (light blue) is performed in the absence of extracellular magnesium (Mg^{2+}-free). In this case, tetrodotoxin (TTX) is added to prevent aberrant plasticity due to spontaneous activity. This type of stimulation typically induces rapid, strong initial growth (peak phase), after which the spine volume stabilizes at a lower level (plateau phase). **(Right)** In paired protocols, two-photon glutamate uncaging (light blue) is paired with depolarization (in voltage clamp by increasing the holding potential, or in current clamp by inducing a backpropagating action potential). Paired stimulation typically leads to a gradual growth of the dendritic spine over time. AMPA receptors in the spine head are depicted in red. The dashed gray lines reflect that the correlation between stimulation protocol and temporal profile of spine growth is not absolute.

electrode. In voltage clamp experiments, the cell is depolarized (typically to 0 mV) while glutamate is uncaged at a spine (Matsuzaki et al., 2004; Harvey and Svoboda, 2007; Lee et al., 2009). In current clamp experiments, current is injected to induce action potential firing while glutamate is uncaged at a spine (Tanaka et al., 2008; Hayama et al., 2013). Alternatively, all-optical uncaging LTP experiments can be performed by pairing optogenetically induced postsynaptic depolarization with glutamate uncaging (Zhang et al., 2008).

The majority of studies have used magnesium-free protocols, which has the great advantage that electrical access to the postsynaptic cell is not required and the studied neuron can be left unperturbed. However, performing experiments in magnesium-free extracellular solution is far from physiological: NMDA receptors are constantly "primed" for activation and addition of TTX is required to block all spontaneous electrical activity. Furthermore, the absence of magnesium could affect several other cellular processes that require magnesium (de Baaij et al., 2015). Paired protocols mimic physiological conditions more accurately. Under physiological conditions, the magnesium block will be relieved by depolarization of the postsynaptic membrane (Gambino et al., 2014). An additional advantage is that the use of patch clamp electrophysiology allows recording of the uncaging-induced excitatory postsynaptic current (uEPSC).

This way, the laser power can be tuned to induce uEPSCs with amplitudes that are similar to single synapse EPSCs (typically ∼10–20 pA) to mimic synaptic glutamate levels (Matsuzaki et al., 2001, 2004; Harvey and Svoboda, 2007; Steiner et al., 2008; Lee et al., 2009; Hill and Zito, 2013). However, the use of electrophysiology makes paired protocols more invasive. Signaling molecules that are required for LTP may "wash out" while perfusing the cell with internal solution from the patch pipette, thereby reducing or abolishing the ability to induce LTP (Malinow and Tsien, 1990; Matsuzaki et al., 2004; Tanaka et al., 2008).

In conclusion, the choice of protocol involves several practical and biological considerations, such as the need for patch clamp electrophysiology, washout of signaling molecules, and resemblance of the physiological situation. It is important to realize that these protocols are not completely interchangeable: in the next section, we will discuss how the induction protocol may affect the magnitude and temporal profile of LTP expression.

EXPRESSION OF SINGLE SYNAPSE LTP

Inducing LTP in a synapse has two major effects: the number of postsynaptic AMPA receptors is increased and the spine volume is enlarged. After LTP, a presynaptic stimulus will induce a postsynaptic current with larger amplitude than before. This is largely due to an increase of AMPA receptors in the postsynaptic membrane (Kessels and Malinow, 2009; Huganir and Nicoll, 2013; Moretto and Passafaro, 2018). Matsuzaki et al. (2004) showed that also in single potentiated spines, the AMPA receptor-mediated currents increase within minutes after stimulation. Many LTP induction paradigms, such as high-frequency stimulation, theta burst stimulation and optical stimulation of afferents lead to persistent spine growth, which was shown by fluorescence imaging (Lang et al., 2004; Okamoto et al., 2004; De Roo et al., 2008; Wiegert et al., 2018) and electron microscopy (Van Harreveld and Fifkova, 1975; Buchs and Muller, 1996; Bourne and Harris, 2011). *In vivo*, spine volumes fluctuate and spines are continuously formed and removed (Caroni et al., 2012; Berry and Nedivi, 2017). Spine dynamics are enhanced after experience and are thought to support long-lasting changes in neural circuits during experience-dependent plasticity (Holtmaat et al., 2006; Hofer et al., 2009; Roberts et al., 2010). For instance, specific spines grow during a motor learning task, and inducing shrinkage of these spines disrupts the acquired motor skill (Hayashi-Takagi et al., 2015). Spine growth is largely attributed to remodeling of actin, which is highly enriched in spines. When a spine is potentiated, polymerization of actin in the spine head leads to more filamentous actin and a bigger spine (Matsuzaki et al., 2004; Okamoto et al., 2004; Harvey et al., 2008; Bosch and Hayashi, 2012; Nakahata and Yasuda, 2018). These morphological changes (actin polymerization and spine growth) and functional changes (increase in AMPA receptors) are often correlated but might be regulated independently.

To monitor the expression of LTP in individual synapses, the increase in ampltidue of the uEPSC can be quantified. The uEPSC at a spine can go up 40–120% within minutes after LTP induction (Matsuzaki et al., 2004; Harvey and Svoboda, 2007; Steiner et al., 2008; Tønnesen et al., 2014). While quantifying uEPSC increase is a useful method to assess functional LTP, it can be technically challenging. Other than on the strength of the synapse, the uEPSC amplitude also depends on the laser power at the uncaging location, the local caged glutamate concentration, and the distance of the uncaging spot to the postsynaptic density, all of which are challenging to keep stable at growing spines during the experiment. Furthermore, electrical access to the postsynaptic cell is required. Quantification of the morphological changes of the spine head is therefore often used as an alternative measure.

Spine growth can be quantified using two-photon microscopy images of the stimulated spine over time. Depending on the initial size and the protocol used, spine heads show (transient) growth up to 200–400% (Matsuzaki et al., 2004; Bosch et al., 2014; Tønnesen et al., 2014; Murakoshi et al., 2017). Spine size correlates strongly with synapse strength under resting conditions *in vitro* (Matsuzaki et al., 2001; Noguchi et al., 2005; Zito et al., 2009) and *in vivo* (Noguchi et al., 2011). Because of this strong correlation, as well as the technical challenges of quantifying uEPSC amplitude over time, spine growth is often taken as a proxy for functional LTP. The correlation between size and function is, however, not absolute: morphological and functional changes might not match perfectly in the first hour after LTP induction (Bosch et al., 2014; Meyer et al., 2014), and functional LTP can also occur in the absence of spine head growth (Araya et al., 2014). It is important to mention that the high laser power used for glutamate uncaging can induce photodamage and swelling of the spine head when the laser beam is aimed too close to the spine head. Swelling due to photodamage could potentially confound actual spine growth due to LTP but can be prevented by aiming the laser beam ∼0.5–1 μm away from the spine head.

Other Morphological Changes

The increase in spine head size is not the only morphological change upon LTP. Several studies have reported shorter and/or thicker spine necks after LTP induction (Tanaka et al., 2008; Araya et al., 2014; Bosch et al., 2014; Tønnesen et al., 2014). These changes in spine neck geometry seem to be consistent with an increase in electrical coupling (Araya et al., 2006, 2014; Tønnesen et al., 2014) and may provide a mechanism for synaptic strengthening independent of AMPA receptor regulation. In addition, glutamate uncaging may induce remodeling of the extracellular space, possibly via glial responses (Tønnesen et al., 2018).

Glutamate uncaging bypasses the need of activating glutamate release of the presynaptic terminal and allows isolation of the postsynaptic component of LTP. However, the presynaptic bouton is probably also affected by glutamate uncaging. After a putative LTP-inducing uncaging protocol, boutons increase their size by ∼50% gradually over the course of 1–3 h, maintaining the correlation between bouton size and spine size (Meyer et al., 2014).

It has also been reported that repeated glutamate uncaging on the dendrite can induce the formation of a new dendritic spine at the uncaging location within seconds, which can become

functional within 30 min (Kwon and Sabatini, 2011; Hamilton et al., 2012). In a different study it was shown that new spines rapidly mature and become functional (Zito et al., 2009). New spines have the capacity to grow upon glutamate uncaging, which significantly increases their persistence (Hill and Zito, 2013).

Variability in Spine Growth

There is a remarkable level of variability in the reported time course and magnitude of spine growth between studies, even within the same brain region and cell type (Tanaka et al., 2008; Bosch et al., 2014). Many studies report an initial peak (or transient phase) of a few minutes in which the spine grows drastically. This peak growth can range from 100 to 400%. This phase is then followed by a plateau (or sustained phase) where the spine growth declines and stabilizes, typically at 50–100% (Harvey and Svoboda, 2007; Tanaka et al., 2008; Patterson et al., 2010; Bosch et al., 2014; Tønnesen et al., 2014; Oh et al., 2015; Harward et al., 2016). Other studies report a gradual spine growth over the course of 5–10 min, which then stabilizes at a plateau, without a significant peak (Harvey and Svoboda, 2007; Tanaka et al., 2008; Hayama et al., 2013; Hu et al., 2019). Even when comparing studies that show a similar temporal pattern of spine growth, peak and plateau magnitudes often vary significantly. One could wonder to what extent extreme peak spine growth resembles the physiological situation.

Technical differences such as differences in quantification methods and model systems could partially explain this remarkable variability, but other factors may be more vital. First of all, the initial size of the spine before induction of LTP matters: small spines have a larger growing capacity than spines that are already larger to begin with (Matsuzaki et al., 2004; Tanaka et al., 2008). It has even been suggested that the large spines cannot grow upon stimulation at all (Matsuzaki et al., 2004; Tanaka et al., 2008). Although it is difficult to compare initial spine size between studies, a difference in initial spine size may explain some of the observed differences in spine growth magnitude.

More importantly, the choice of LTP induction protocol will crucially affect the magnitude and time course of spine growth. This was first observed by Tanaka et al. (2008). When they paired glutamate uncaging with backpropagating action potentials, it led to a gradual growth of the spine, reaching close to 150% growth. However, when they performed glutamate uncaging in absence of extracellular magnesium, spine growth showed an initial peak in which spine volume reached twofold growth (100%), after which spine growth declined to reach a plateau phase at 50% (Tanaka et al., 2008). Similarly, Harvey and Svoboda (2007) reported a gradual spine growth of 80% when using a paired protocol. A similar magnesium-free protocol resulted in 175% peak growth, declining to a plateau at 75% growth (Harvey and Svoboda, 2007). These studies clearly suggest that different induction protocols activate different intracellular signaling pathways, resulting in differences in spine growth. Typically, magnesium-free induction protocols lead to peak-plateau growth, while paired protocols often induce gradual spine growth (**Figure 1**; although this correlation is not absolute, Matsuzaki et al., 2004; Zhang et al., 2008; Lee et al., 2009).

Why do different induction protocols lead to such remarkable differences in spine growth? The choice of induction protocol likely affects how downstream signaling pathways are activated. This already occurs at the level of calcium concentration elevation, the key signal for LTP. While calcium influx is typically restricted to the spine head in magnesium-free stimulation protocols, paired protocols also cause an increase of calcium concentration in the dendritic shaft (see below). This differential spatial calcium profile may also lead to differential activation of downstream signaling molecules, and it is interesting to speculate how these could be linked to the peak and plateau phases of spine growth. For instance, the study by Tanaka et al. (2008) showed that the paired protocol involved BDNF signaling and protein synthesis to induce spine growth, while spine growth was independent of BDNF in the magnesium-free protocol. However, a more recent study observed that BDNF also affects spine growth after a magnesium-free protocol (Harward et al., 2016). These data suggest that there is not a single universal mechanism for the expression of LTP in spines. Multiple modes of LTP may exist, and different protocols may activate different signaling mechanisms. We will discuss these signaling pathways in the next sections. We will first describe which pathways are activated when LTP is induced in single spines, followed by a discussion on how signaling pathways between nearby synapses can interact.

SINGLE SYNAPSE LTP SIGNALING PATHWAYS

In this section, we discuss the signaling pathways that are activated when a single spine is potentiated. Expression of LTP has been extensively examined using chemical or electrical LTP induction, in which multiple synapses are activated in many neurons simultaneously and signaling pathways are triggered in a large part of the neuron. These studies have established that calcium influx through NMDA receptors and subsequent activation of CaMKII are essential for LTP. Downstream signaling pathways eventually lead to actin remodeling and the insertion of AMPA receptors, resulting in a stronger synapse. Here we limit our discussion to studies using two-photon glutamate uncaging to induce LTP in a single synapse. By inducing LTP in a single synapse, it is possible to study the activation of molecules in LTP signaling pathways with the highest temporal and spatial detail.

Glutamate Receptors

Glutamate uncaging on a dendritic spine activates AMPA receptors and NMDA receptors in the postsynaptic density (although glutamate receptors can also be found extrasynaptically and presynaptically, Parsons and Raymond, 2014; Bouvier et al., 2018). AMPA receptors mainly conduct sodium and potassium ions and are largely responsible for synaptic membrane depolarization in the spine. Binding of glutamate to NMDA receptors is usually not sufficient to open the channel, as they are blocked by magnesium. Only when the postsynaptic membrane is sufficiently depolarized, during AMPA receptor activation, a backpropagating action potential or a dendritic spike, the

magnesium block is relieved and NMDA channels open. When NMDA receptors are activated, it leads to the rapid influx of calcium ions through the channel pore into the dendritic spine. Many studies have demonstrated that NMDA receptor activation is required for the growth of single spines (Matsuzaki et al., 2004; Harvey and Svoboda, 2007; Zhai et al., 2013; Tang and Yasuda, 2017).

Not all spines contain both AMPA receptors and NMDA receptors. AMPA receptor content is correlated to spine size, and the smallest spines can be silent, meaning that they contain no AMPA receptors and therefore no current can be measured when the spine is exposed to glutamate. These silent spines however do contain NMDA receptors (Béïque et al., 2006; Busetto et al., 2008). This allows these spines to undergo LTP by growing and recruiting AMPA receptors.

Besides ionotropic glutamate receptors, dendritic spines also contain group I metabotropic glutamate receptors (mGluRs). These mGluRs are enriched immediately next to the postsynaptic density (Scheefhals and MacGillavry, 2018). When glutamate uncaging is performed at a dendritic spine, it is likely that mGluRs will also be activated, especially when long uncaging pulses or many repetitions are used. When the metabotropic glutamate receptors are blocked during the induction of single synapse LTP, spine growth typically remains intact (Matsuzaki et al., 2004; Zhai et al., 2013; Bosch et al., 2014; Colgan et al., 2018), suggesting they do not play a major role in LTP induction.

Calcium

Calcium entering the spine via NMDA receptor activation is considered the key signal to trigger LTP. During a single synapse LTP induction protocol, each uncaging stimulus leads to a brief influx of calcium into the dendritic spine (Lee et al., 2009; Zhai et al., 2013; Colgan et al., 2018). There is a tight inverse correlation between spine head volume and calcium levels: uncaging on a smaller spine leads to a higher calcium concentration (Noguchi et al., 2005; Sobczyk et al., 2005). This can partly be explained by geometric differences, but different subunit composition of NMDA receptors in smaller spines may also play a role (Sobczyk et al., 2005). Depending on the geometry of the spine neck (length and width), some calcium will diffuse from the spine head into the dendritic shaft (Noguchi et al., 2005; Zhai et al., 2013).

While calcium influx through NMDA receptors is crucial for LTP induction, other sources of calcium can be involved as well. When glutamate uncaging is paired with postsynaptic depolarization, voltage-gated calcium channels (VGCCs) in the dendrite and spine get activated (Lee et al., 2009; Müllner et al., 2015) and this will lead to additional calcium influx. An experiment by Zhai et al. (2013) suggests that VGCCs do not play a role in the induction of LTP under magnesium-free conditions, but may affect the plateau level of spine growth.

Calcium-Sensing Proteins

The increase of calcium concentration upon NMDA receptor activation is sensed by Calcium/calmodulin kinase II (CaMKII), and activation of CaMKII is essential for the induction of LTP. CaMKII can associate with several structures in the spine head, such as filamentous actin and several proteins in the postsynaptic density (Okamoto et al., 2004; Hell, 2014; Kim et al., 2015). Changes in local CaMKII levels may occur after single synapse LTP induction. CaMKII concentration in the spine has been reported to temporarily drop for 5 min (Bosch et al., 2014), or to slightly but persistently increase after LTP induction (Zhang et al., 2008). As changes in the concentration of CaMKII are also dependent on changes in spine volume, it is important to mention that these studies use different induction protocols (magnesium-free versus paired) and observe a different temporal pattern and amplitude of spine growth. Both studies agree that the total amount of bound (as opposed to freely diffusing) CaMKII in the spine head increases after LTP induction (Zhang et al., 2008; Bosch et al., 2014). It was previously shown that the amount of bound CaMKII in the spine correlates strongly with spine size and uEPSC amplitude under baseline conditions (Asrican et al., 2007), suggesting that the trapping of CaMKII in the spine head is directly related to strengthening of the spine during LTP. On longer timescales, the fraction of bound CaMKII returns to baseline (Asrican et al., 2007; Zhang et al., 2008), indicating that unbound CaMKII slowly diffuses to the spine to restore the ratio of bound/unbound CaMKII.

CaMKII is activated by calcium and the calcium-binding protein calmodulin. Calmodulin associates with and dissociates from CaMKII within seconds. The association of calmodulin and CaMKII does not accumulate during a single synapse LTP induction protocol (Chang et al., 2019). CaMKII activation however does increase with every uncaging pulse, thereby integrating multiple calcium signals. CaMKII even stays active for up to 1 min after the end of the induction protocol (Lee et al., 2009; Chang et al., 2017, 2019). This accumulation and persistence of the signal can be explained by autophosphorylation (at the threonine 286 residue), allowing CaMKII to remain active after calcium/calmodulin unbinds. Autophosphorylation of CaMKII is important for LTP induction: the slower inactivation rate permits signal integration at relatively low frequency stimulation. Only at extremely high frequencies (>8 Hz) can repeated stimulation sustain CaMKII activation without autophosphorylation (Chang et al., 2017).

CaMKII plays an important role in spine growth. Multiple studies show that pharmacological inhibition or genetic knockout of CaMKII strongly reduces the plateau phase of spine growth, while peak growth is maintained (Matsuzaki et al., 2004; Lee et al., 2009; Murakoshi et al., 2011; Hedrick et al., 2016; Incontro et al., 2018; Saneyoshi et al., 2019). Using a photoactivatable CaMKII inhibitor, Murakoshi et al. (2017) demonstrated that CaMKII activation is required for only 1 min during LTP induction. Interestingly, both the peak and plateau of spine growth were strongly reduced when the inhibitor was activated during the entire LTP induction protocol. When the inhibitor was activated 30 s after the start of the induction protocol, only plateau growth was reduced while peak growth remained (Murakoshi et al., 2017). These data suggest that the peak and plateau growth require different durations of CaMKII activation but are in disagreement with experiments using pharmacological inhibition of CaMKII (discussed above).

The spatial extent of CaMKII activation depends on the LTP induction protocol. In a typical magnesium-free induction protocol, CaMKII activation is mostly restricted to the spine head (Lee et al., 2009), although a small amount of active CaMKII might be found in the dendritic shaft (Chang et al., 2017). However, when glutamate uncaging is paired with postsynaptic depolarization, dendritic VGCCs are activated and as a result CaMKII is also strongly activated in the dendritic shaft (Lee et al., 2009).

In addition to CaMKII, the phosphatase calcineurin (CaN) is also activated in the spine head and dendritic shaft when calcium levels increase. While CaMKII is sensitive to both the frequency and number of uncaging stimuli, CaN is less sensitive to stimulation frequency and mainly responds to the number of stimuli (Fujii et al., 2013). Calcineurin activity is typically associated with spine shrinkage and synaptic depression (Zhou et al., 2004; Hayama et al., 2013; Nabavi et al., 2013; Oh et al., 2015).

GTPases: Ras, RhoA, Cdc42, Rac1

During and after LTP induction, several small GTPases are activated in the dendritic spine via both CaMKII-dependent and -independent pathways. Small GTPases are enzymes that often function as "molecular switches" in biological signaling pathways and play an important role in regulating the synaptic actin cytoskeleton and plasticity (Hotulainen and Hoogenraad, 2010; Patterson and Yasuda, 2011). Harvey et al. (2008) used a FRET-sensor to show that the small GTPase Ras is activated in the dendritic spine within 1 min after glutamate uncaging. Activity decays substantially in 5 min, but some Ras stays activated for at least 15 min. Ras activation is partly dependent on CaMKII (Harvey et al., 2008), likely through phosphorylation of the Ras GTPase activating protein SynGAP (Araki et al., 2015), but Ras activation also depends on PI3K and PKC activity (Harvey et al., 2008). Ras presumably acts via the extracellular signal-regulated kinase ERK via the Ras-MEK pathway. ERK activation in the spine peaks within 5 min after LTP induction and lasts for 20 min (Tang and Yasuda, 2017). Ras-ERK signaling plays an important role in spine growth: interfering with Ras activation or with its downstream Raf-MEK-ERK pathway reduces the magnitude of the plateau, but not of the peak spine growth (Harvey et al., 2008; Zhai et al., 2013). When both CaMKII and the Ras-Raf-MEK-ERK pathway are inhibited, plateau spine growth is almost completely abolished, suggesting that these pathways together are responsible for the majority of spine growth in the plateau phase (Harvey et al., 2008).

RhoA, a member of the Rho subfamily of GTPases, is also activated in the stimulated spine within 30 s upon LTP induction. While the level of activity largely decays within 5 min, some activity remains for 30 min. Another Rho GTPase family member, Cdc42, shows similar activation kinetics. RhoA and Cdc42 activation is partially dependent on CaMKII signaling. Functional LTP is completely abolished when RhoA or Cdc42 are inhibited. Inhibition of RhoA or its downstream effector Rock reduces both the peak phase and the plateau phase of spine growth, while interfering with Cdc42 or its downstream effector Pak affects plateau phase spine growth only (Murakoshi et al., 2011). Experiments by Hedrick et al. (2016) suggest that Cdc42 activation can be downstream from autocrine BDNF signaling (see below), while RhoA is activated independently.

A third Rho GTPase family member Rac1 is also activated rapidly in the dendritic spine upon LTP induction, partly in a CaMKII- and BDNF-dependent manner. Rac1 shows stronger sustained activation than RhoA and Cdc42. Interfering with Rac1 signaling significantly reduces both the peak phase and plateau phase of spine growth (Hedrick et al., 2016). Recently, it was shown that sustained activation of Rac1 is regulated by the guanine nucleotide exchange factor Tiam1. Tiam1 forms a complex with activated CaMKII, and both proteins reciprocally keep each other active. Interfering with Tiam1 or the complex formation between CaMKII and Tiam1 significantly affects spine growth (Saneyoshi et al., 2019).

Together, the picture emerges that glutamate uncaging induces spine growth and functional LTP via multiple, and partially overlapping, GTPase pathways (Nakahata and Yasuda, 2018).

PKC and PKA Signaling

Classical protein kinase C (PKC) family proteins are typically activated in the presence of calcium and the lipid diacylglycerol (DAG) (Lipp and Reither, 2011). It has been shown that specifically PKCα mediates the plateau phase of spine growth (Colgan et al., 2018). PKC activation occurs in the dendritic spine and is extremely rapid: PKC is activated after every uncaging pulse, but activity has already decayed by the time of the next uncaging pulse (at 0.5 Hz). Blocking calcium influx through NMDA receptors completely abolishes PKC activation, and PKC activation and spine growth are reduced when the production of DAG by Phospholipase C (PLC) is inhibited. During LTP induction, PLC is activated by autocrine BDNF-TrkB signaling (see below) and not by mGluR activation (Colgan et al., 2018).

Another important kinase, protein kinase A (PKA), seems to play a modulatory role in LTP (Esteban et al., 2003; Blitzer et al., 1998; Man et al., 2007). PKA activity depends on cyclic AMP levels and is downstream of a variety of G-protein coupled receptors. Single synapse LTP induction leads to rapid activation of PKA in the spine, which decays back to baseline in 5 min. Interestingly, PKA activation was found to be downstream of NMDA receptor activation (Tang and Yasuda, 2017). LTP does not require PKA activation, but PKA activation can boost single synapse LTP (Govindarajan et al., 2011; Yagishita et al., 2014). However, PKA activation originating from a single stimulated spine may not be sufficient for this boosting effect, and more global PKA activation, for instance via dopaminergic neuromodulatory signals (Yagishita et al., 2014), may be required.

Actin

Actin is the major structural component of the dendritic spine, and spine growth requires actin remodeling. Matsuzaki et al. (2004) already showed that single spine growth is prevented in the presence of Latrunculin A, a drug that sequesters actin monomers and prevents actin polymerization. In resting conditions, two pools of actin can be found in the dendritic spine: a highly

dynamic pool located at the tip of the spine head and a very stable pool at the base of the spine. After LTP induction, a third "enlargement" pool appears, and this pool seems to be responsible for spine growth (Honkura et al., 2008).

Upon LTP induction, the amount of actin in the spine and several actin-interacting proteins (Arp2/3, profilin, Aip1, drebrin, α-actinin, cofilin) increases in parallel with spine growth (Bosch et al., 2014). Some of these proteins (Arp2/3, Aip1, actin, cofilin) increase rapidly during peak growth, and the concentration of cofilin in the spine head remains elevated for at least 30 min. Upon LTP induction, cofilin is phosphorylated by LIM kinase, which is downstream of the Cdc42-Pak and RhoA-Rock pathways discussed above (Bosch et al., 2014). Phosphorylation of cofilin is required for the peak and plateau phases of spine growth (Noguchi et al., 2016). In the first few minutes, phosphorylated cofilin presumably severs actin filaments and thereby boosts the nucleation of new actin filaments and branching by Arp2/3, resulting in spine growth. After this initial phase, cofilin is dephosphorylated again and can decorate actin filaments, thereby stabilizing them. In absence of cofilin, the plateau phase of spine growth is abolished (Bosch et al., 2014).

Interestingly, during baseline conditions CaMKII associates with actin filaments in the spine head. When calcium flows into the spine head and activates CaMKII, autophosphorylation of CaMKII causes it to dissociate from filamentous actin, allowing binding of cofilin and other actin regulators to remodel the actin cytoskeleton. After dephosphorylation, CaMKII quickly binds and thereby stabilizes actin filaments. It has been suggested that the rapid and transient (∼1 min time window) dissociation of CaMKII from filamentous actin allows the rapid and transient peak spine growth observed in some studies (Kim et al., 2015). Preventing CaMKII F-actin dissociation strongly reduces functional LTP in slices and strongly reduces fear learning *in vivo* (Kim et al., 2015, 2019).

AMPA Receptors and Postsynaptic Density

Within minutes after single synapse LTP induction, synaptic strengthening is expressed as an increase in the amount of AMPA receptors on the spine surface (Makino and Malinow, 2009; Patterson et al., 2010; Bosch et al., 2014; Chiu et al., 2017; Soares et al., 2017) and can be measured by an increase in AMPA receptor-mediated currents (Matsuzaki et al., 2004; Harvey and Svoboda, 2007; Steiner et al., 2008; Tønnesen et al., 2014). The increase of AMPA receptors in the postsynaptic density involves receptor phosphorylation (Boehm et al., 2006) and mainly occurs via lateral diffusion in the membrane, but exocytosis of AMPA receptor-containing vesicles also contributes (Makino and Malinow, 2009; Patterson et al., 2010; Chiu et al., 2017; Choquet, 2018). A local increase of exocytosis rate occurs during LTP induction, which seems partially dependent on Ras-ERK-mediated, but CaMKII-independent, pathways. CaMKII signaling is likely involved in anchoring of AMPA receptors to spines (Patterson et al., 2010).

The postsynaptic density (PSD) consists of a cluster of proteins close to the postsynaptic membrane. Important PSD proteins such as PSD95, Homer and Shank act as a scaffold to position and anchor ionotropic and metabotropic glutamate receptors (Scheefhals and MacGillavry, 2018). Remodeling of the PSD during LTP is a complex, multi-step process. Under basal conditions, the size of the PSD strongly correlates with the size of the spine head. After LTP induction, the postsynaptic density increases in size, but components arrive in the spine with a delay compared with the rapid AMPA receptor insertion (Steiner et al., 2008; Bosch et al., 2014; Meyer et al., 2014). In some spines, transient spine growth can be observed after glutamate uncaging, which returns to baseline after ∼2 h without any changes to the PSD (Meyer et al., 2014). After successful single synapse LTP, it takes at least 1 h for the correlation between PSD and spine size to restore (Bosch et al., 2014; Meyer et al., 2014).

Protein Synthesis

Spine growth can occur in the absence of protein synthesis (Harvey and Svoboda, 2007; Harward et al., 2016), but some single synapse LTP induction protocols require synthesis of new proteins. Tanaka et al. (2008) showed that when single synapse LTP is induced in low extracellular magnesium, spine growth is independent of protein synthesis. However, when a similar induction protocol is paired with postsynaptic spiking in physiological levels of magnesium, spine growth is strongly dependent on protein synthesis (Tanaka et al., 2008). A more recent study showed that spine growth induced under magnesium-free conditions actually does require protein synthesis, but only more than 30 min after LTP induction. This study also shows that the gradual recruitment of the postsynaptic scaffolding protein Homer1b was abolished when protein synthesis was inhibited (Bosch et al., 2014). Another study also showed that protein synthesis is involved in the maintenance of enlarged spines after LTP induction. Govindarajan et al. (2011) showed that spine growth returns to baseline after 2 h, but spine growth could be maintained by pharmacological activation of PKA in the entire slice. This maintenance depended on protein synthesis. When glutamate uncaging was paired with PKA activation in the absence of protein synthesis, spine growth was entirely prevented (Govindarajan et al., 2011). These studies illustrate that protein synthesis may be important for spine growth and functional LTP at the single synapse level under certain circumstances, but it is not clear how exactly it is triggered and when it is required.

Brain-Derived Neurotrophic Factor (BDNF) Signaling

The neurotrophic factor BDNF has been shown to affect single synapse LTP. Tanaka et al. (2008) suggested that BDNF is released after pairing glutamate uncaging with postsynaptic spiking, but not after glutamate uncaging in magnesium-free conditions. However, Harward et al. (2016) observed that a similar uncaging protocol in magnesium-free conditions does lead to rapid release of BDNF from the stimulated spine, and that this is partially dependent on CaMKII activation. BDNF release resulted in rapid and sustained activation of the BDNF receptor TrkB in the stimulated spine, the dendrite and neighboring spines

(Harward et al., 2016). BDNF, via TrkB activation, may promote small GTPase and PKC activation (Hedrick et al., 2016; Colgan et al., 2018). In the Tanaka study, LTP was shown to require protein synthesis, while in the Harward study spine growth was independent of protein synthesis. These studies and others (Bosch et al., 2014) suggest that (autocrine) BDNF signaling can facilitate, but is not absolutely required for, single synapse LTP. They also show that subtle differences in stimulation protocol may lead to remarkable differences and illustrate our limited understanding of under which conditions BDNF is released from dendrites and spines.

Spine Shrinkage

While we focus here on potentiation of spines, glutamate uncaging has also been used to induce shrinkage of spines and depression of synaptic transmission. Low frequency uncaging at a single spine (90 pulses at 0.1 Hz, paired with depolarization) can induce spine shrinkage, which is accompanied by a decrease in uEPSC amplitude. The shrunken spines can undergo LTP and grow again when exposed to an LTP stimulus. There is an interesting difference between small and large spines: while large spines require mGluR and IP3 receptor activation to shrink, the small spines do not (Oh et al., 2013). Spine shrinkage is dependent on non-ionotropic signaling of NMDA receptors, as it can occur without calcium flux through NMDA receptor channels. Surprisingly, a stimulation protocol that normally induces single synapse LTP leads to spine shrinkage when NMDA receptor-dependent calcium flow is inhibited, revealing that NMDA receptors may activate both pathways in parallel (Stein et al., 2015). We refer interested readers to a more elaborate discussion of the molecular mechanisms involved in spine shrinkage and elimination (Stein and Zito, 2018).

Multiple Parallel Pathways

Studies on the induction of LTP in individual dendritic spines have revealed the temporal and spatial activation patterns of signaling molecules and pathways during LTP induction and expression. Single synapse LTP involves several, partially overlapping, intracellular signaling pathways, and the time course and magnitude of single synapse LTP is critically shaped by the molecular pathways involved. It will be important to gain a better understanding into the stimuli that trigger the different signaling pathways and how multiple pathways interact within single spines and their direct vicinity.

The majority of studies use a magnesium-free protocol to assure NMDA receptor activation during the stimulation protocol, and signaling pathways with this protocol have been described in great detail (Nishiyama and Yasuda, 2015; Nakahata and Yasuda, 2018). Under physiological conditions, glutamate receptor activation coincides with postsynaptic depolarization during LTP induction, which likely affects the spatial and temporal dynamics of signaling molecules in the stimulated spine and adjacent dendrite. Indeed, in a direct comparison, very different patterns of CaMKII activation were observed in magnesium-free and paired protocols (Lee et al., 2009). In addition, the requirements for protein synthesis and the contribution of BDNF signaling were found to be highly protocol-dependent (Tanaka et al., 2008; Govindarajan et al., 2011). This supports the idea that the spatiotemporal activation patterns of downstream signaling pathways are inevitably shaped by the induction protocol. This is important to realize, as experimental conditions are never fully representative of the *in vivo* physiological conditions. To interpret the intricate signaling pathways in the proper context, it is key to improve our understanding of how and when they are evoked at the single synapse level *in vivo*.

INTERACTIONS BETWEEN SYNAPSES

In the previous section we discussed the signaling pathways that can be activated when a single synapse undergoes LTP. Dendrites are tightly packed with hundreds of dendritic spines, and neighboring spines may influence each other. Under physiological conditions, single synapse activation may be rare and multiple synapses are receiving inputs simultaneously. It is therefore important to consider how adjacent synapses can influence each other's plasticity.

Crosstalk

Harvey and Svoboda were the first to use glutamate uncaging to show crosstalk can occur between single spines during LTP induction: spines that received a weak ("subthreshold," 1 ms uncaging pulse) stimulus did not undergo LTP, but they only showed LTP when a nearby spine was stimulated with a strong (4 ms uncaging pulse) LTP-inducing stimulus. It is not clear whether the difference in pulse duration reflects a difference in the level and/or duration of NMDA receptor activation, or a difference in the type of glutamate receptors that are activated. The spine that received the weak stimulus showed the same level of spine growth and functional LTP as the spine that received the strong stimulus (**Figure 2A**). This crosstalk occurs over a timescale of several minutes and a length scale of 5–10 μm, both in magnesium-free and paired protocols (Harvey and Svoboda, 2007).

Several signaling molecules that are activated during LTP induction can diffuse out of the stimulated spine and affect signaling in neighboring spines. While calcium influx and CaMKII activation are brief and mostly restricted to the dendritic spine (when using a magnesium-free induction protocol) (Harvey et al., 2008; Lee et al., 2009; Otmakhov et al., 2015), their downstream effectors are often active on longer time scales and spread over longer distances. This has been studied mostly for the GTPases. After single synapse LTP induction, the GTPases Ras and Rac1 diffuse freely over approximately 10 μm within the dendrite and neighboring spines, while RhoA reaches ∼5 μm (Harvey et al., 2008; Murakoshi et al., 2011; Hedrick et al., 2016). Although Cdc42 is equally mobile as its family members, Cdc42 activation is contained within the spine head (Murakoshi et al., 2011).

Diffusion of these signaling molecules can reduce the threshold for LTP in neighboring spines and thereby mediate synaptic crosstalk. When Ras signaling is pharmacologically inhibited, crosstalk is reduced (Harvey et al., 2008). Similarly,

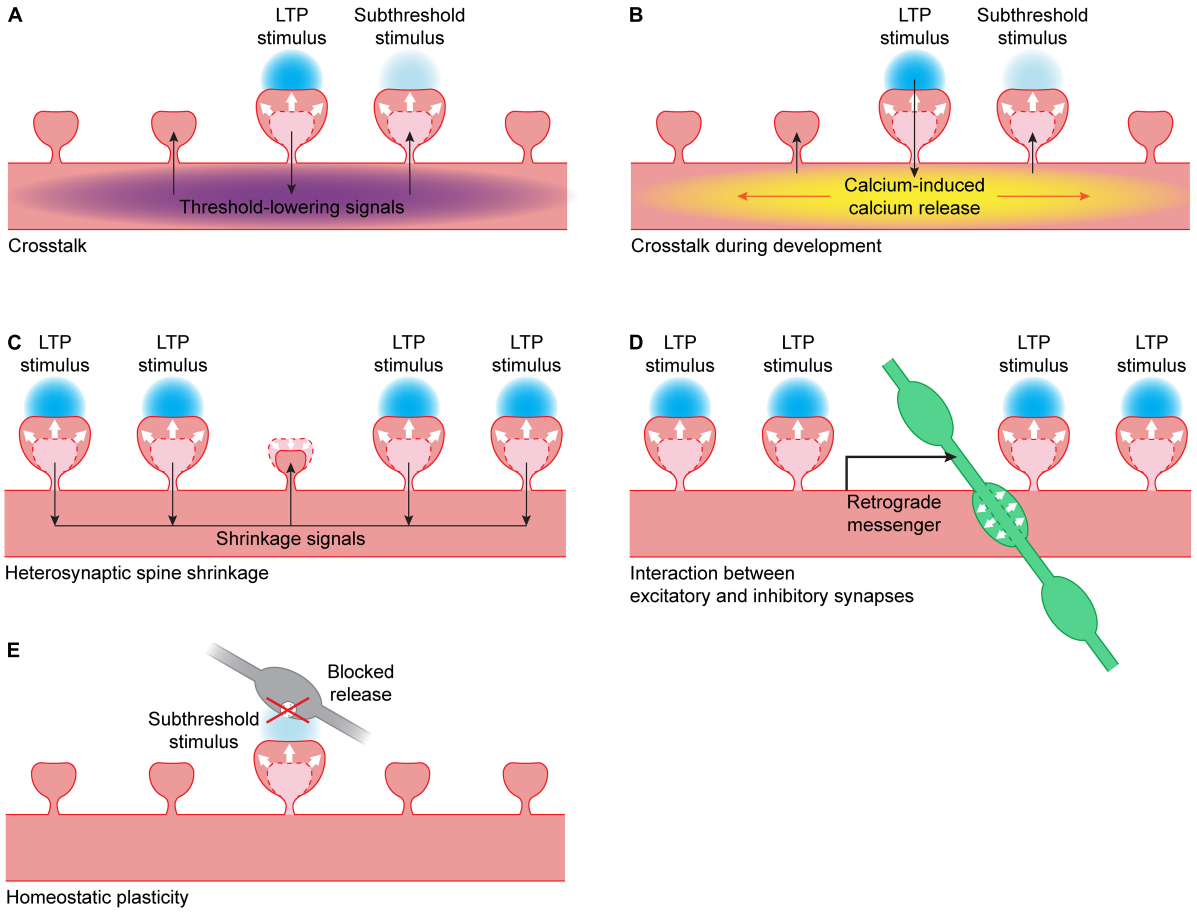

FIGURE 2 | Interactions between synapses. **(A)** When LTP is induced in a single spine using glutamate uncaging (blue), this leads to the spread of threshold-lowering signals (purple) in the dendrite. When a nearby spine receives a stimulus that is normally subthreshold, spine growth will occur. Threshold-lowering signals include the small GTPases Ras, Rac1 and RhoA (Harvey et al., 2008; Murakoshi et al., 2011; Hedrick et al., 2016) and BDNF-TrkB signaling (Harward et al., 2016; Colgan et al., 2018). PKA and ERK activity also spreads over the dendrite but it is unclear if these kinases are able to lower the LTP threshold (Zhai et al., 2013). **(B)** During development, the calcium influx in a single spine during glutamate uncaging can trigger calcium-induced calcium release (yellow). This leads to propagating calcium waves in the dendrite, and a nearby spine receiving a stimulus that is normally subthreshold will now show spine growth (Lee et al., 2016). **(C)** When a cluster of spines undergo LTP, this can lead to the activation of shrinkage signals. These signals can induce shrinkage of an unstimulated dendritic spine nearby (Oh et al., 2015). **(D)** When a cluster of spines undergo LTP, this can lead to the production of a retrograde messenger by the postsynaptic neuron. This messenger can trigger the growth of a presynaptic inhibitory bouton (green) nearby (Hu et al., 2019). **(E)** When vesicle fusion in the presynapse (gray) has been blocked for a prolonged period of time, this can lead to a lowering of the LTP threshold: when a spine receives a stimulus that is normally subthreshold, it will show spine growth (Lee et al., 2010).

interfering with the spread of Rac1 and RhoA activity out of suprathreshold spine significantly reduces crosstalk without affecting the growth of the suprathreshold spine (Hedrick et al., 2016). A subthreshold stimulus (using shorter glutamate pulses) does not activate Ras and only weakly activates Rac1 and RhoA. A suprathreshold stimulus on a spine nearby can elevate Ras, Rac1 and RhoA activation levels in the subthreshold spine above threshold. Cdc42 activation is similar after subthreshold and suprathreshold stimuli (Harvey et al., 2008; Hedrick et al., 2016).

During single synapse LTP, activation of PKC is almost completely restricted to the stimulated dendritic spine. However, when a nearby spine receives a subthreshold stimulus at the same time, PKC also gets activated in the subthreshold spine. PKC activation is triggered by fast and local calcium influx through NMDA receptors but is also sensitive to DAG production through TrkB-PLC signaling (Colgan et al., 2018). Because TrkB activation slowly spreads over a stretch of 10 μm (Harward et al., 2016), PKC may integrate the activation history of nearby spines (Colgan et al., 2018).

Both PKA and ERK activation spread over more than 10 μm of dendrite and invade nearby spines, with PKA showing a sharper spatial gradient and a more rapid decay than ERK (Tang and Yasuda, 2017). ERK can stay active for a long time and diffuse over long distances within the dendritic tree. LTP induction on at least 3 spines on two different branches within 30 min leads to sustained nuclear ERK activation that is likely mediated by diffusion of activated ERK from the stimulated spines. Nuclear ERK activation is dependent on mGluR activation and may

require PKC to maintain ERK activation. In the nucleus, ERK likely activates transcription factors that are responsible for the late phase of LTP (Zhai et al., 2013).

While the crosstalk described above typically works on a time scale of a few minutes (Harvey and Svoboda, 2007), another form of crosstalk has been observed on longer time scales. In the study by Govindarajan et al. (2011), glutamate uncaging alone leads to spine growth that returns to baseline after 2 h, while combining glutamate uncaging with pharmacological PKA activation leads to protein synthesis-dependent LTP that lasts for at least 4 h. Interestingly, when a spine is exposed to glutamate uncaging alone before or after a neighboring spine is exposed to glutamate uncaging paired with PKA activation, both spines grow persistently for up to 4 h. This crosstalk works over a time range of tens of minutes (both pre and post) and tens of micrometers on the same dendritic branch, and depends on protein synthesis (Govindarajan et al., 2011).

Besides biochemical interactions, nearby spines will also interact electrically. Their postsynaptic potentials summate, often in non-linear ways (London and Häusser, 2005; Losonczy and Magee, 2006; Tran-Van-Minh et al., 2015). For instance, it was shown that when four spines on a distal dendritic segment are stimulated, calcium levels in individual spines are higher than when the spines are activated individually, and this is mediated by NMDA receptors. Simultaneous subthreshold stimulation at these spines (in the presence of magnesium and without depolarization) can overcome the LTP threshold and can induce functional LTP in these spines (Weber et al., 2016).

Together, these studies show that single synapse LTP is affected by the recent activity of nearby spines and mediated by many factors, such as local kinase activity and dendritic exchange of GTPases (Nishiyama and Yasuda, 2015; Yasuda, 2017). Crosstalk of LTP between neighboring spines along the same dendrite is particularly relevant *in vivo*, where synapses with similar properties or activity patterns often cluster together (Kleindienst et al., 2011; Makino and Malinow, 2011; Bloss et al., 2016, 2018; Wilson et al., 2016; Iacaruso et al., 2017).

Plasticity and Crosstalk During Development

During development, the rules for synaptic plasticity and crosstalk are not the same as in mature neurons (Lohmann and Kessels, 2014). When uncaging at a single spine in young, developing neurons, calcium is less restricted in the spine head than in mature neurons, and calcium influx through NMDA channels can be boosted by calcium-induced calcium release (CICR) (Lee et al., 2016). Activating individual spines often leads to propagating calcium waves in the dendrite that are mediated by CICR from intracellular stores. However, propagating calcium waves after LTP induction have not been observed in more mature neurons, suggesting that the coupling between NMDA receptors and internal calcium stores is developmentally regulated. In young neurons, all spine growth depends on CICR, suggesting that calcium influx through NMDA receptors is not sufficient to induce LTP in young neurons. When a strong stimulus on one spine is paired with a weaker stimulus on a neighboring spine, this leads to sustained spine growth in both spines, and this crosstalk is also dependent on CICR (**Figure 2B**). In general, the high level of local crosstalk in young neurons suggests the clustered maturation of spines. Indeed, it was shown that mature synapses, which have high AMPA/NMDA ratios, tend to cluster together on dendrites of young neurons (Lee et al., 2016).

Heterosynaptic Spine Shrinkage

When a small cluster of spines (at least four) is potentiated using glutamate uncaging, it can induce shrinkage and loss of AMPA receptors at an unstimulated spine close to that cluster (**Figure 2C**) (Oh et al., 2015). This heterosynaptic shrinkage is dependent on the calcium sensing protein calcineurin, mGluR and IP3 receptor signaling, but it is independent of the classical LTP protein CaMKII. When CaMKII is inhibited, spine growth at the stimulated spines is prevented but the unstimulated spine still shrinks. When calcineurin is inhibited, only growth of the stimulated spines remains. This shows that the spine is not shrinking because of competition for resources, but because it is actively being regulated (Oh et al., 2015).

Spine shrinkage can also be induced by combining single spine glutamate uncaging with activation of dendritic $GABA_A$ receptors (Hayama et al., 2013). Neighboring spines within 15 μm also undergo shrinkage, and synaptic transmission is weakened. This type of spine shrinkage depends on NMDA receptor and calcineurin signaling but is independent of mGluR signaling. While shrinkage spreads over the dendrite, a neighboring spine receiving a potentiating stimulus can still overcome the shrinkage signals and grow (Hayama et al., 2013). Together, these studies show that parallel signaling pathways for spine growth and shrinkage exist within the dendrite.

Interaction Between Excitatory and Inhibitory Synapses

Inhibitory synapses are important regulators of dendritic signals. They interact with excitatory synaptic inputs electrically, and they play an important role in regulating calcium dynamics in the dendrite (Higley, 2014). An individual inhibitory synapse can reduce the influx of calcium during a backpropagating action potential locally within the dendrite (Müllner et al., 2015) or even within a single spine (Chiu et al., 2013). Additionally, activation of metabotropic $GABA_B$-receptors reduces NMDA receptor-mediated calcium influx in single activated spines (Chalifoux and Carter, 2010). Inhibitory synapses are therefore likely able to interfere with nearby single synapse LTP induction. It needs to be noted that most studies discussed in this review use MNI-glutamate as their caged compound, which has been shown to have strong antagonistic effects on $GABA_A$ receptors (Fino et al., 2009; Matsuzaki et al., 2010; Ellis-Davies, 2019). In addition, the presence of TTX in experiments using magnesium-free induction protocols also abolishes spontaneous activity in inhibitory neurons. Inhibitory synaptic signaling might therefore be largely blocked in these studies, which may affect the induction and/or expression of single synapse LTP.

Vice versa, LTP at spines also affects nearby inhibitory synapses. Chemical and electrical LTP studies have shown that NMDA receptor activation affects gephyrin clusters and the surface expression of $GABA_A$ receptors (Marsden et al., 2007; Petrini et al., 2014; Flores et al., 2015) and leads to strengthening of inhibitory inputs (Bourne and Harris, 2011; Chiu et al., 2018). Using glutamate uncaging, our lab has recently shown that activation of a cluster of excitatory synapses can trigger the growth of a new inhibitory presynaptic bouton onto the stimulated dendrite via NMDA receptors and a retrograde endocannabinoid signal (**Figure 2D**) (Hu et al., 2019). Such a local coordination mechanism between excitatory and inhibitory plasticity will be important in regulating a balance between excitatory and inhibitory synapses within a dendritic branch and ensuring local inhibitory control over an active excitatory cluster.

Interaction With Homeostatic Plasticity

Homeostatic plasticity operates over long time scales to maintain neuronal network function (Turrigiano, 2012). Neurons can regulate their own excitability by different mechanisms, including synaptic scaling of AMPA receptors (Turrigiano et al., 1998). Although the intracellular signaling pathways underlying synaptic scaling are not entirely clear, it is not unlikely that they partially overlap, or even interfere, with single synapse LTP. Lee et al. (2010) performed single synapse LTP at spines with silent (e.g., tetanus toxin expressing) presynaptic terminals, which had undergone synaptic scaling. They showed that presynaptic silencing leads to a decrease in LTP threshold, such that a stimulus protocol that is normally subthreshold can induce spine growth and functional LTP at presynaptically silenced spines (**Figure 2E**). They did not observe a difference in LTP when a suprathreshold stimulus was used (Lee et al., 2010). This suggests that homeostatic plasticity at individual synapses can affect the threshold for inducing spine growth and LTP.

Similarly, a recent study by Hobbiss et al. (2018) shows that when action potentials are blocked in a hippocampal slice for 48 h using TTX, spines become bigger and stronger, indicative of synaptic scaling. Using glutamate uncaging, the authors showed that small spines that were exposed to TTX treatment grow more after an LTP stimulus than untreated spines of the same size. In addition, a weak stimulus that does not induce sustained spine growth under control conditions induces significant spine growth in the TTX condition. This suggests that homeostatic scaling enhances the capacity to undergo LTP (Hobbiss et al., 2018). However, in an earlier study by Soares et al. (2017), no differences were observed in uncaging-induced spine growth between control and TTX-treated conditions.

CONCLUDING REMARKS

Since the first study reported LTP of a single dendritic spine using glutamate uncaging (Matsuzaki et al., 2004), several protocols have been used to induce single synapse LTP: magnesium-free protocols that do not require electrical access to the postsynaptic neuron or paired protocols attempting to resemble physiological activation of the postsynaptic neuron. The expression of LTP in a single synapse is measured by quantifying the increase in uEPSC amplitude and/or in spine size, which are highly correlated with one another. Thanks to tremendous technological advances, signaling pathways involved in single synapse LTP are studied with spectacularly high spatial and temporal resolution. Remarkably, these studies at the single synapse level revealed that synapses do not necessarily operate individually. Specific signaling proteins leave the spine head and penetrate the dendritic shaft and nearby spines, where they can reduce the threshold for LTP. This implies that the activation and plasticity history of the synapse itself, as well as the history of synapses in its direct dendritic vicinity, strongly influence its capacity to undergo plasticity.

While we gained significantly more insight into the mechanisms of LTP at the single synapse level over the past 15 years, several questions remain and new questions emerge. There is sufficient evidence to conclude that different induction protocols trigger different signaling pathways and lead to different "modes" or levels of LTP expression. Morphological changes (peak and plateau spine growth) and functional LTP (receptor insertion) are not always perfectly aligned and may be evoked via different molecular routes with different experimental induction protocols. It will be the next challenge to understand if these parallel LTP pathways matter under physiological circumstances.

Another major challenge for the field is to understand the systems that are in place to coordinate the multitude of synaptic inputs within the neuron. Synapses with similar properties tend to cluster together on the same dendritic branch (Kleindienst et al., 2011; Druckmann et al., 2014; Bloss et al., 2016, 2018; Wilson et al., 2016; Iacaruso et al., 2017). One could therefore argue that *in vivo*, LTP rarely happens at isolated synapses but perhaps more often at small clusters of co-active synapses. It is therefore important to understand how spines undergoing LTP can interact within dendrites. Several studies have now started to address the mechanisms behind different forms of crosstalk. Expanding these studies to larger clusters of synapses, and including excitatory as well as inhibitory synapses, will allow us to examine under which circumstances synapses cooperate and when they compete for resources.

Research has focused on LTP in single spines, but the current understanding of synaptic depression and shrinkage of dendritic spines is much more limited. Only two uncaging protocols are known to induce LTD in the stimulated spine, and one of those also requires GABA uncaging (Hayama et al., 2013; Oh et al., 2013). Spine shrinkage and synaptic depression are not regulated by the inverse of LTP pathways but involve specific signaling. It will be important for future research to further unravel the spatial and temporal profile of LTD-associated signals and to examine overlap and interaction with LTP pathways.

In recent years, caged GABA compounds became available for two-photon uncaging. While uncaging GABA has been used to identify and quantify the presence of GABA receptors (Kantevari et al., 2010; Kanemoto et al., 2011; Chiu et al., 2013; Villa et al., 2016; Kwon et al., 2018) and to induce nascent excitatory or inhibitory synapses in young neurons (Oh et al., 2016), two-photon GABA uncaging has yet to enter the realm of synaptic

plasticity. It would be interesting to use GABA uncaging to assess changes in the strength of individual inhibitory synapses. Coordination between excitation and inhibition, which is crucial for the proper functioning of neurons, is regulated at the synaptic level (Liu, 2004; Chen et al., 2012, 2015; Bloss et al., 2016; Hu et al., 2019). We therefore expect that improving our understanding of the interaction of excitatory and inhibitory plasticity at the level of single synapses and dendrites, for example by combining two-photon uncaging of glutamate and GABA (Kantevari et al., 2010), will provide us with exciting new insights.

The dendritic branch can be considered the fundamental electrical and biochemical functional unit of the nervous system (Branco and Häusser, 2010; Govindarajan et al., 2011; Lovett-Barron et al., 2012). Single synapse LTP studies are revealing that the molecular signaling pathways underlying single synapse LTP are not limited to the stimulated spine, but kinases, GTPases and other regulators can travel and interact with proteins in the dendrite and neighboring synapses. The precise effect of synaptic activation depends therefore on the activation and plasticity history of the involved synapse as well as excitatory and inhibitory synapses in its direct vicinity. Therefore, synaptic plasticity should always be considered within the context of the local dendritic homeostasis.

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Both authors wrote and revised the manuscript.

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MINI REVIEW
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Intra- and Extracellular Pillars of a Unifying Framework for Homeostatic Plasticity: A Crosstalk Between Metabotropic Receptors and Extracellular Matrix

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In the face of chronic changes in incoming sensory inputs, neuronal networks are capable of maintaining stable conditions of electrical activity over prolonged periods of time by adjusting synaptic strength, to amplify or dampen incoming inputs [homeostatic synaptic plasticity (HSP)], or by altering the intrinsic excitability of individual neurons [homeostatic intrinsic plasticity (HIP)]. Emerging evidence suggests a synergistic interplay between extracellular matrix (ECM) and metabotropic receptors in both forms of homeostatic plasticity. Activation of dopaminergic, serotonergic, or glutamate metabotropic receptors stimulates intracellular signaling through calmodulin-dependent protein kinase II, protein kinase A, protein kinase C, and inositol trisphosphate receptors, and induces changes in expression of ECM molecules and proteolysis of both ECM molecules (lecticans) and ECM receptors (NPR, CD44). The resulting remodeling of perisynaptic and synaptic ECM provides permissive conditions for HSP and plays an instructive role by recruiting additional signaling cascades, such as those through metabotropic glutamate receptors and integrins. The superimposition of all these signaling events determines intracellular and diffusional trafficking of ionotropic glutamate receptors, resulting in HSP and modulation of conditions for inducing Hebbian synaptic plasticity (i.e., metaplasticity). It also controls cell-surface delivery and activity of voltage- and Ca^{2+}-gated ion channels, resulting in HIP. These mechanisms may modify epileptogenesis and become a target for therapeutic interventions.

Keywords: mGluRs, extracellular matrix, HCN channels, SK channels, AMPARs, ADAMTS, dopamine receptors, 5-HT7 receptors

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INTRODUCTION

Homeostatic plasticity enables neurons to stabilize network activity within an optimal dynamic range over prolonged periods of time, thereby playing a fundamental neuroprotective role during pathological conditions that tend to alter function and integrity of neuronal networks. Homeostatic plasticity entails negative feedback mechanisms that can alter diverse aspects of network function: the number of shared connections, the strength of excitatory and inhibitory synaptic transmission, the excitatory/inhibitory ratio [phenomena collectively designated as

homeostatic synaptic plasticity (HSP)] and the level of intrinsic excitability [homeostatic intrinsic plasticity (HIP)] (Silberberg et al., 2005; Hahn et al., 2019). Recent evidence suggests that many of these homeostatic mechanisms are not always active but instead are triggered by behavioral states, such the sleep–wake rhythm, and by modulatory neurotransmitters and metabotropic receptors, such as the glutamatergic, dopaminergic, and serotonergic receptors (Tononi and Cirelli, 2014; Hengen et al., 2016; Diering et al., 2017).

In addition to extensive analyses of ion channel trafficking and intracellular signaling pathways involved in the different forms of homeostatic plasticity, several studies have revealed the importance of synaptic extracellular matrix (ECM) molecules, such as neuronal activity-regulated pentraxin (Narp) (Chang et al., 2010), and major ECM receptors such as β3 integrin (Cingolani et al., 2008; McGeachie et al., 2012). More recently, the attention was drawn also to the hyaluronic-acid-based perisynaptic ECM (Korotchenko et al., 2014; Valenzuela et al., 2014), incorporating lecticans, link proteins, and tenascin-R (Ferrer-Ferrer and Dityatev, 2018). Here, we review emerging common themes linking ECM remodeling with other major mechanisms of homeostatic plasticity, which are intriguingly "clustered" around regulation of metabotropic receptors.

mGluRs IN HOMEOSTATIC SYNAPTIC PLASTICITY

Metabotropic glutamate receptors (mGluRs) represent a prominent family of class C G protein-coupled receptors (GPCRs). These receptors assemble into constitutive dimers with each subunit comprising a "Venus flytrap" domain, a large extracellular N-terminal domain that contains the endogenous ligand-binding site (Pin and Bettler, 2016). Based on sequence homology, G-protein coupling, and ligand selectivity, we can distinguish three major groups of mGluRs. In neurons, group I mGluRs (mGluR1 and 5) are enriched in postsynaptic compartments where they couple to $G\alpha_q$ heterotrimeric G proteins and activate phospholipase C. Group II (mGluR2 and 3) and III mGluRs (mGluR4, 6, 7, and 8) are instead localized mainly presynaptically where they couple to $G\alpha_{i/o}$ and inhibit adenylyl cyclase (Niswender and Conn, 2010).

Group I mGluRs are involved in the induction of both Hebbian and homeostatic forms of synaptic plasticity. The mechanism of activation of these receptors in the two forms of plasticity is, however, different. In Hebbian mGluR-induced long-term depression (mGluR-LTD), mGluR1/5 are activated by synaptically released glutamate; consequently, only mGluR1/5 localized in close proximity to the activated synapses will contribute to weakening of synaptic transmission in a synapse-specific manner (Oliet et al., 1997; Luscher and Huber, 2010). Conversely, in homeostatic synaptic downscaling, mGluR1/5 are activated by the immediate early gene Homer1a, which is induced in response to the increase in network activity. Rather than being synapse specific, Homer1a induction is cell wide and promotes mGluR1/5 activity in a glutamate-independent manner by disrupting the scaffold formed by the constitutively expressed long forms of Homer, which firmly anchor mGluR1/5 at perisynaptic sites (Ango et al., 2001; Hu et al., 2010). Because disruption of mGluR1/5 clusters favors constitutive activation of these receptors, Homer1a acts effectively as an endogenous mGluR1/5 allosteric modulator. It is noteworthy that, albeit different in the induction mechanism, mGluR-LTD and homeostatic synaptic downscaling eventually converge as both forms of synaptic plasticity induce tyrosine dephosphorylation of GluA2 subunits of AMPA-type glutamate receptors (AMPARs), with a consequent increase in the internalization rate of GluA2-containing AMPARs (**Figure 1**; Moult et al., 2006; Gladding et al., 2009; Scholz et al., 2010; Jang et al., 2015). Interestingly, these mechanisms appear to be relevant to synapse remodeling and memory consolidation during sleep because the synaptic levels of Homer1a are dramatically increased during sleep, leading to loss of synaptic mGluR5, constitutive activation of these receptors, and weakening of synapses (Diering et al., 2017).

RECIPROCAL INTERACTIONS BETWEEN EXTRACELLULAR ENVIRONMENT AND mGluRs IN HOMEOSTATIC SYNAPTIC PLASTICITY

Functional characterization of mGluRs has focused predominantly on proteins involved in intracellular scaffolding and signaling (O'Connor et al., 2014). However, it is becoming increasingly clear that mGluRs also associate with cell adhesion molecules (CAMs) and ECM components and that these interactions play a crucial role in regulating localization and signaling of mGluRs. Recently, group III mGluRs have been shown to interact with ELFN1 [extracellular leucine-rich repeat (LRR) and fibronectin type III domain-containing 1 (Tomioka et al., 2014; Cao et al., 2015; Wang et al., 2017; Dunn et al., 2018)], a member of the family of LRR CAMs, which play an essential role in specifying synaptic connectivity (de Wit and Ghosh, 2016). The interaction likely involves the glutamate-binding domains on mGluRs and the N-terminal LRR protein-interaction domain on ELFN1 (Stachniak et al., 2019). In the hippocampus and cortex, ELFN1 is found exclusively in somatostatin interneurons from where it interacts transsynaptically with presynaptic mGluR7 expressed in pyramidal neurons, thereby recruiting mGluR7 selectively at synapses between pyramidal neurons and somatostatin interneurons (Tomioka et al., 2014). The enrichment of mGluR7 is responsible for reducing neurotransmitter release probability and for endowing these synapses with their distinctive short-term facilitation properties (Sylwestrak and Ghosh, 2012). Similarly, in the retina, transsynaptic interaction between ELFN1 and mGluR6 plays an essential role in retaining mGluR6 at the synapses between rods and bipolar cells (Cao et al., 2015; Wang et al., 2017). These observations exemplify the relevance of extracellular interactions for clustering mGluRs at synapses.

Perhaps more importantly, recent work suggests that ELFN1 has not only a structural role, but it could also promote

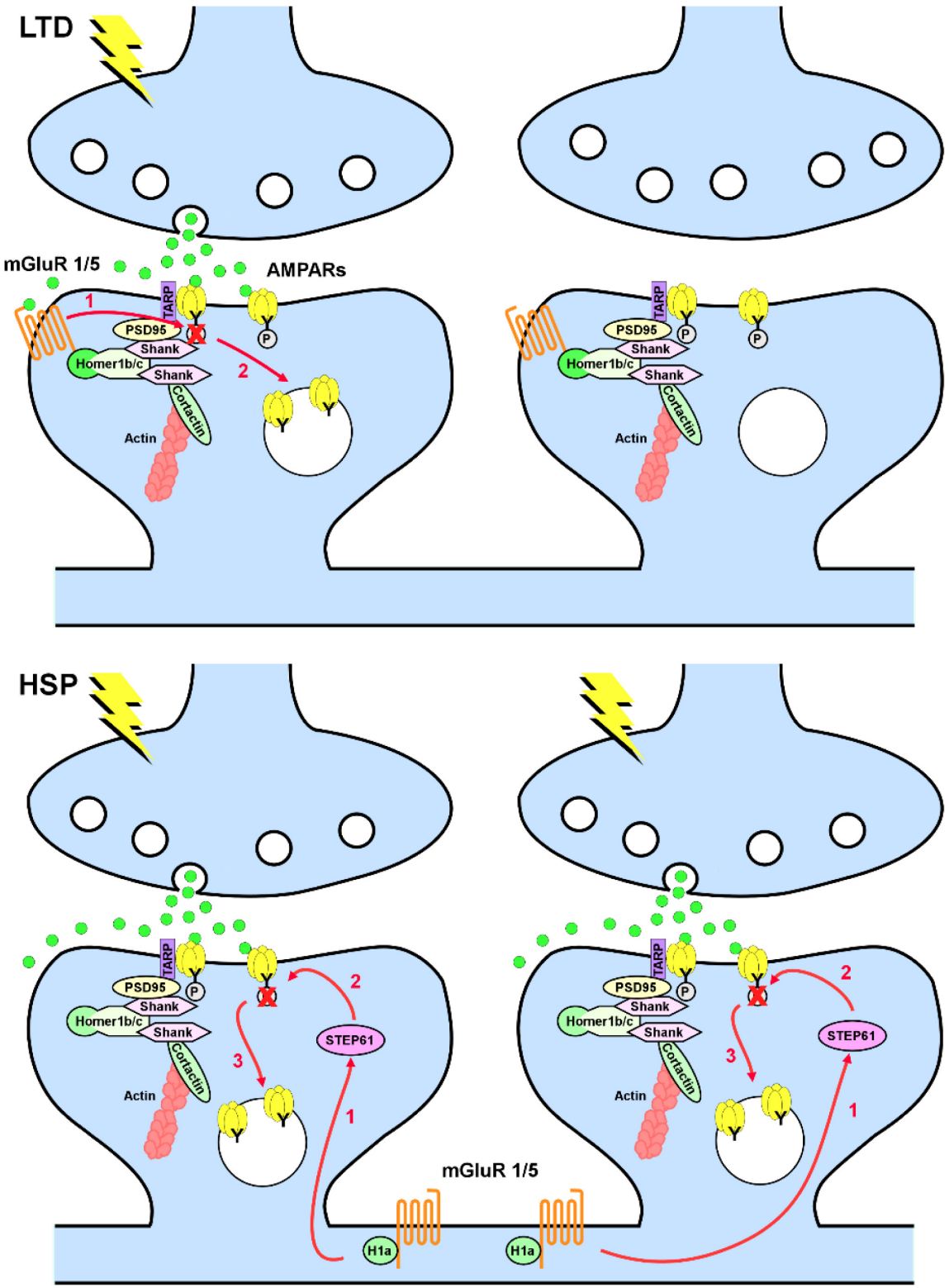

FIGURE 1 | Metabotropic glutamate receptors 1/5 (mGluR1/5) in long-term depression (LTD) and homeostatic synaptic plasticity (HSP). Top, in LTD, mGluR1/5 are anchored at perisynaptic sites via Homer 1b/c and activated in a synapse-specific manner by synaptically released glutamate. Activation of mGluR1/5 leads to tyrosine dephosphorylation of the GluA2 subunit of AMPARs (1), with consequent increase in AMPAR endocytosis (2). Bottom, in HSP induced by chronic increase in network activity, induction of Homer1a decouples mGluR1/5 from the synaptic signaling machinery and induce a constitutive glutamate-independent activation of mGluR1/5. Homer1a-induced mGluR1/5 signaling requires upregulation of the striatal-enriched protein tyrosine phosphatase (STEP61; 1), with consequent dephosphorylation of the GluA2 subunit of AMPARs (2) and increase in AMPAR endocytosis (3) in a non-synapse-specific manner.

constitutive activation of group III mGluRs. Specifically, the interaction between ELFN1 and group III mGluRs may favor dimerization of these receptors and stabilize them in a constitutive active conformation (Dunn et al., 2018; Stachniak et al., 2019). In this model, postsynaptic ELFN1 would act therefore as an endogenous allosteric modulator to bias group III mGluR activity from a glutamate-induced to a tonic-signaling mode. This dual role of ELFN1 as scaffold protein and allosteric modulator is closely reminiscent of the well-characterized function of Homer proteins in regulating localization and basal activity of group I mGluRs in homeostatic plasticity (Ango et al., 2001; Hu et al., 2010; Shim et al., 2016). Crucially, the interplay between ELFN1 and mGluR7 is physiologically relevant because loss of either proteins induces similar phenotypes in mice, specifically hyperactivity and increased susceptibility to seizures (Sansig et al., 2001; Dolan and Mitchell, 2013; Tomioka et al., 2014).

The interplay between ECM and mGluRs is twofold: on the one hand, the extracellular environment controls mGluRs, as exemplified above, but on the other hand, the signaling through mGluRs modulates the extracellular environment. For instance, stimulation of group I mGluRs activates the disintegrin metalloproteinase tumor necrosis factor-α-converting enzyme (TACE; alias, ADAM 17), which in turn cleaves the membrane protein neuronal pentraxin receptor (NPR). This process, known as "shedding," induces the release of a soluble ectodomain of NPR, which coclusters the pentraxin Narp and AMPARs through extracellular interactions, and stimulates AMPAR endocytosis. Remarkably, this mechanism is relevant for both hippocampal and cerebellar mGluR-LTD, which rely otherwise on divergent signaling pathways (Cho et al., 2008).

Although it is not known whether similar signaling pathways are engaged in homeostatic plasticity, it is worth noting that one of the best-studied substrates of TACE is tumor necrosis factor alpha (TNF-α), which is required for inactivity-induced HSP both *in vitro* and *in vivo* (Stellwagen and Malenka, 2006; Kaneko et al., 2008). TNF-α increases surface expression of β3 integrin, which interacts directly with the GluA2 subunit of AMPARs and is required for regulating network activity and HSP but not mGluR-LTD (Cingolani et al., 2008; McGeachie et al., 2012; Pozo et al., 2012; Jaudon et al., 2019). In addition, under conditions of hyperactivity, expression and secretion of the pentraxin Narp is rapidly and dramatically upregulated, which promotes clustering and retention of AMPARs on parvalbumin-expressing interneurons, thus increasing excitatory inputs to these cells, which culminates in homeostatic upregulation of principal cell inhibition (Chang et al., 2010). Accordingly, $Narp^{-/-}$ mice display increased sensitivity to kindling-induced seizures.

METABOTROPIC RECEPTOR-DRIVEN ECM REMODELING AND HOMEOSTATIC SYNAPTIC PLASTICITY

Like TACE-induced extracellular proteolysis is important for downregulation of excitatory transmission, disintegrin and metalloprotease with thrombospondin motifs (ADAMTS)-mediated proteolytic modifications of ECM are associated with inactivity-induced homeostatic synaptic upscaling (Valenzuela et al., 2014). Using an antibody specific for a brevican fragment cleaved by the matrix metalloproteases ADAMTS4 and 5, the researchers revealed perisynaptic brevican processing by these proteases. Interestingly, after induction of homeostatic plasticity in neuronal cell cultures by prolonged network inactivity, there is an increased brevican processing at inhibitory as well as excitatory synapses, corresponding to the ADAMTS4 subcellular localization. This study suggests therefore a permissive role of perisynaptic ECM remodeling in removing inhibitory constrains of synaptic growth necessary for synaptic upscaling.

Which factors control the activity of ADAMTS and other extracellular proteases and hence the integrity of perisynaptic ECM? Recent findings implicate dopaminergic and serotonergic neuromodulation. Activation of D1-type dopamine (DA) receptors induces proteolysis of brevican and aggrecan via ADAMTS4 and 5 specifically at excitatory synapses of rat cortical neurons (Mitlöhner et al., 2019). Pharmacological inhibition and short hairpin RNA-mediated knockdown of ADAMTS4 and 5 reduces brevican cleavage. The study further demonstrates that synaptic activity and DA neuromodulation are linked to ECM rearrangements via increased cAMP levels, NMDA receptor (NMDAR) activation, and signaling via protein kinase A (PKA) and the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII). These findings are in line with the previously reported increase in the extracellular activity of the tissue plasminogen activator (tPA) protease after activation of D1-like DA receptors via a PKA-dependent pathway (Ito et al., 2007). Strikingly, tPA may directly activate ADAMTS4 (Lemarchant et al., 2014), suggesting that at least partially elevated remodeling of perisynaptic ECM may be due to tPA-ADAMTS4 processing. Previous analysis of tPA function in homeostatic plasticity had revealed a bidirectional effect of tPA on the composition of the postsynaptic density (PSD) (Jeanneret and Yepes, 2017). In inactive neurons, tPA induces phosphorylation and accumulation of pCaMKIIα in the PSD, resulting in pCaMKIIα-induced phosphorylation and synaptic recruitment of GluA1-containing AMPARs. In active neurons, tPA drives pCaMKIIα and pGluA1 dephosphorylation and subsequent removal from the PSD. These effects require active NMDARs and cyclin-dependent kinase 5 (Cdk5)-induced phosphorylation of the protein phosphatase 1 (PP1). Thus, tPA, and hence ADAMTS4 and potentially other members of the ADAMTS family, may act as homeostatic regulators of the postsynaptic efficacy in a CaMKII-dependent manner. In addition, enzymatic digestion of highly sulfated forms of heparan sulfates with heparinase I was reported to induce homeostatic synaptic upscaling in association with upregulated phosphorylation of CaMKII in cultured hippocampal neurons (Korotchenko et al., 2014). This is noteworthy, as heparan sulfate proteoglycans are major components of the ECM and play key roles in misfolding, oligomerization, and fibrillation of amyloidogenic proteins, stabilization of protein aggregates, as well as for cellular uptake of proteopathic seeds during their spreading (Maiza et al., 2018).

In contrast to DA, serotonin (5-HT) induces ECM remodeling by activating the matrix metalloproteinase 9 (Bijata et al., 2017). This study revealed a physical interaction between 5-HT7 receptors and CD44, the major receptor of the neural ECM backbone, hyaluronic acid. 5-HT7 receptor stimulation increases local matrix metalloproteinase 9 activity, which leads to CD44 cleavage and Cdc42 activation, followed by an increase in neuronal outgrowth and elongation of dendritic spines. Although there is no experimental evidence that this signaling may induce homeostatic plasticity, hyaluronic acid is known to control activity of postsynaptic L-type Ca^{2+} channels (Kochlamazashvili et al., 2010), which have been implicated in inactivity-induced HSP (Thiagarajan et al., 2005). Indeed, enzymatic digestion of hyaluronic acid leads to epileptiform activity *in vitro* (Vedunova et al., 2013), and mice deficient in hyaluronic acid synthase HAS3 show epileptic seizures (Arranz et al., 2014).

mGluRs IN HOMEOSTATIC INTRINSIC PLASTICITY

Homeostatic adaptation of neuronal firing following prolonged changes in sensory inputs can be achieved not only by adjusting synaptic strength, to amplify or dampen incoming inputs (i.e., HSP), but also by altering intrinsic excitability (i.e., HIP). Observed initially in primary cortical cultures in response to the same pharmacological manipulations that induce HSP (Desai et al., 1999), HIP has been shown to contribute to network stability of various brain regions *in vivo*, often in cooperation with HSP (Debanne et al., 2019). As for HSP, both sensory deprivation and elevated network activity, as observed in status epilepticus, can induce HIP (Maffei and Turrigiano, 2008; Kirchheim et al., 2013; Kuba et al., 2015; Milshtein-Parush et al., 2017). Although the molecular mechanisms and ion channels that contribute to stabilizing intrinsic excitability vary widely according to the brain region and neuron type considered, much attention has been given to hyperpolarization-activated, cyclic nucleotide-gated (HCN) and K^+ channels. Here, we will consider the contribution of HCN channels and of a subclass of K^+ channels, the small-conductance Ca^{2+}-activated K^+ channels (SK channels) to HIP, and their interplay with metabotropic signaling and ECM.

HCN Channels

Hyperpolarization-activated, cyclic nucleotide-gated channels, whose family comprises four members (HCN1, 2, 3, and 4), are of special interest because they are activated by membrane hyperpolarization, but they mediate a mixed Na^+ and K^+ current (I_h), whose net effect is depolarizing. This means that opening (and closing) of HCN channels will counteract membrane hyperpolarization (and depolarization), thereby stabilizing membrane potential. Crucially, this negative-feedback regulation occurs also in the subthreshold range because HCN channels are partially open at voltages near the resting membrane potential (Biel et al., 2009). HCN channels play also a key role in regulating dendritic integration in CA1 hippocampal and layer V cortical pyramidal neurons. In these neurons, the dendritic density of HCN channels, and most notably of HCN1, increases dramatically along the apical dendrites with distance from the soma. As a consequence of this somato-dendritic gradient, HCN1 effectively dampens excitatory synaptic currents originating in distal apical dendrites, thus limiting their temporal summation (Stuart and Spruston, 2015).

A complex network of cell-autonomous, non-cell-autonomous, and activity-dependent mechanisms regulates distal dendritic targeting of HCN1 in pyramidal neurons. For example, the brain-specific HCN channel auxiliary subunit tetratricopeptide repeat-containing Rab8b-interacting protein (TRIP8b) supports dendritic enrichment of HCN1 via intracellular interactions (Piskorowski et al., 2011). The ECM protein Reelin provides instead a non-cell-autonomous extracellular factor for anchoring HCN1 at distal dendrites. Reelin is a large glycoprotein whose signaling is important for regulating both neuronal positioning during development and synaptic plasticity in the adult brain (Ferrer-Ferrer and Dityatev, 2018). In the adult, it is secreted by a subset of inhibitory interneurons with a non-uniform distribution across the hippocampus and the neocortex. This sets the conditions for establishing gradients of Reelin across these two brain regions. Binding of Reelin to the lipoprotein receptors, the apolipoprotein E receptor type 2 (APOER2) and the very low-density lipoprotein receptor (VLDLR), on pyramidal neurons activates Src family tyrosine kinases and the cytoplasmic signaling molecule Dab1. This signaling pathway promotes Hebbian synaptic plasticity by tyrosine phosphorylation of NMDARs (Beffert et al., 2005) and is required for giving the distal dendritic compartments of CA1 and layer V pyramidal neurons their molecular identity, including the enrichment in HCN1 (Kupferman et al., 2014).

Hyperpolarization-activated, cyclic nucleotide-gated channel expression is also under the control of neuronal activity both *in vitro* (Brager and Johnston, 2007; Shin and Chetkovich, 2007; Arimitsu et al., 2009; Gasselin et al., 2015; Shim et al., 2016; Schanzenbacher et al., 2018) and *in vivo*. Indeed, whisker trimming, to induce sensory deprivation in the barrel cortex, causes a decrease in HCN channel density in the distal region of the apical dendrites of layer V pyramidal neurons (Breton and Stuart, 2009). The network-activity-dependent regulation is bidirectional as pharmacological treatments that increase and decrease network activity up- and downregulate HCN activity, respectively. These adaptations are homeostatic because HCN channels actively oppose excitatory drive. Interestingly, they also play an essential metaplastic role as they counterbalance the complementary changes in synaptic strength that take place following HSP, thus ensuring that the propensity to induce Hebbian long-term potentiation (LTP) does not vary following chronic changes in network activity (Gasselin et al., 2015).

In hippocampal CA1 pyramidal neurons, more proximal apical dendrites are innervated by the Schaffer collateral pathway from CA3 pyramidal neurons, while distal apical dendrites are contacted by the perforant pathway from the entorhinal cortex (Megias et al., 2001). The localization of HCN1 in distal dendrites of CA1 neurons requires the activity of the perforant pathway and opening of NMDARs with subsequent elevation

of intracellular Ca^{2+} and activation of CaMKII (Shin and Chetkovich, 2007). Conversely, activation of group I mGluRs at the Schaffer collateral and downstream activation of PKC downregulates HCN channels (Brager and Johnston, 2007). It is therefore plausible that a differential balance between NMDAR and mGluR1/5 signaling at the Schaffer collateral and perforant pathway synapses (Xu et al., 2010) may contribute to distal HCN enrichment.

As opposed to the situation in pyramidal neurons, HCN channels are uniformly distributed on the dendrites of cerebellar Purkinje cells (Angelo et al., 2007). Furthermore, neuronal activity affects the expression of HCN channels in Purkinje cells and pyramidal neurons in opposite directions. In Purkinje cells, chronic activity deprivation upregulates, rather than downregulating, HCN channels, thus decreasing the excitability of these neurons. Because Purkinje cells are inhibitory, these adaptations have a net homeostatic effect on network function. It is worth noting that HIP in Purkinje cells is initiated by glutamate-independent activation of mGluR1 (Shim et al., 2016), similarly to what happens for HSP in cortical neurons (Hu et al., 2010). Hence, constitutive group I mGluR signaling is important for the induction of both HSP and HIP and may change dramatically neuronal network function and stability. For example, in CA3 hippocampal pyramidal neurons, transient pharmacological stimulation of group I mGluRs appears to switch mGluR1 into a constitutive active state with consequent changes in multiple intrinsic ion conductances [including suppression of the Ca^{2+}-dependent K^+ current mediating the slow afterhyperpolarization (sI_{AHP}) and activation of a voltage-gated cationic, TRPC-like current ($I_{mGluR(V)}$)], which have an overall epileptogenic effect in the hippocampus (Bianchi et al., 2009, 2012; Young et al., 2013).

SK Channels

Small-conductance Ca^{2+}-activated K^+ channels, whose family comprises four members (SK1–4), are voltage-independent K^+ channels broadly expressed in the brain (Stocker and Pedarzani, 2000; Pedarzani and Stocker, 2008; Gymnopoulos et al., 2014). Low concentrations (in the submicromolar range) of intracellular Ca^{2+} activate SK channels by binding to calmodulin, which serves as intrinsic Ca^{2+} sensing subunit. In addition to calmodulin, SK channels interact constitutively with protein kinase CK2 and protein phosphatase 2A, which modulate Ca^{2+} sensitivity (Adelman et al., 2012). Because SK channels hyperpolarize membrane potential in response to intracellular Ca^{2+} rises, they have a well-recognized role in counteracting somatic excitability and Hebbian synaptic plasticity (Lujan et al., 2009). Recent evidence suggests a possible role for SK channels also in homeostatic plasticity. Notably, SK2 channels colocalize and coassemble with mGluR1 and mGluR5 in Purkinje cells and hippocampal pyramidal neurons, respectively (Garcia-Negredo et al., 2014; Lujan et al., 2018). In CA1 hippocampal and layer V cortical pyramidal neurons, stimulation of group I mGluRs activates inositol trisphosphate receptors (IP_3Rs), which support intracellular Ca^{2+} waves in dendrites and somata. While these Ca^{2+} waves often evoke a transient SK-mediated hyperpolarization (Hagenston et al., 2008; El-Hassar et al., 2011), selective pharmacological stimulation of mGluR5 reduces SK currents in layer V pyramidal neurons (Sourdet et al., 2003; Cannady et al., 2017).

Our recent data indicate that the ECM proteoglycan brevican may constitutively inhibit activity of group III mGluRs postsynaptically in CA1 pyramidal neurons (Song et al., 2019). Under conditions of brevican deficiency, these receptors, however, become active and reduce cAMP levels in neurons. This results in inhibition of PKA activity, which normally drives endocytosis of SK channels, and hence in increased cell surface expression of SK channels and reduced excitability of pyramidal neurons. Such mechanism may be induced by activity-dependent proteolysis of brevican and plays therefore a homeostatic role by reducing dendritic neuronal excitability.

HEPARAN SULFATE PROTEOGLYCANS IN AXONAL EXCITABILITY

Similar to dendritic, also axonal excitability is under the control of ECM molecules, which accumulate at the axon initial segment (AIS). Among these molecules are tenascin-R and heparan sulfate proteoglycans of glypican and syndecan subfamilies. Acute treatment of hippocampal slices with heparinase I results in impaired LTP due to a reduction in axonal excitability (Minge et al., 2017). Our recent findings demonstrate elevated CaMKII activity 24 h after intrahippocampal heparinase I injection *in vivo*, which is accompanied by reduced axonal excitability and impaired context discrimination in fear conditioning paradigms (Mironov et al., 2018). These effects appear to be mediated by CaMKII because cotreatment with heparinase I and the CaMKII inhibitor AIP fully rescues neuronal excitability and context discrimination and because the increase in CaMKII expression at the AIS is accompanied by changes in accumulation of ankyrin G. In summary, these data suggest that the CaMKII signaling cascade activated by ECM remodeling is essential for both HSP and the control of axonal excitability. So far, no specific GPCRs have been implicated in the mechanisms linking heparan sulfates to CaMKII. Still, heparan sulfates are known to modulate presentation of diverse positively charged ligands to GPCRs. For instance, they stabilize the formation of chemokine dimers and higher order chemokine oligomers that are required for binding to the G-protein-coupled chemokine receptors. One of these, CXCR4, is activated by chemokine C-X-C motif ligand CXCL12α bound to heparan sulfates (Thakar et al., 2017) and is known to regulate CaMKII activity (Hu et al., 2017).

HOMEOSTATIC PLASTICITY AND METAPLASTICITY

Synaptic and intrinsic homeostatic responses may cooperate with each other to maintain constant conditions for the induction of Hebbian-type synaptic plasticity. A good example is the aforementioned homeostatic regulation of HCN channels. Downscaling of excitatory synaptic currents following chronic

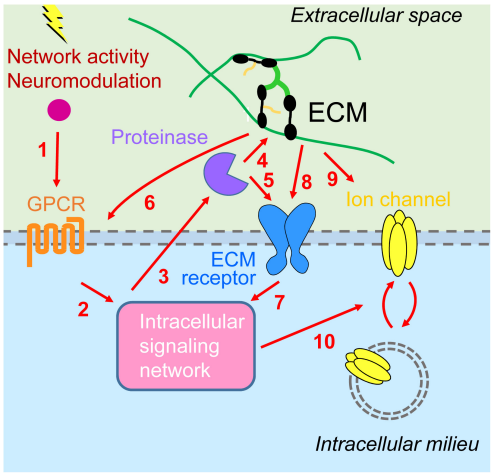

FIGURE 2 | Interplay between metabotropic receptors and extracellular matrix in homeostatic plasticity. (1) Network activity and neuromodulatory systems stimulate G protein-coupled receptors (GPCRs) [metabotropic glutamate receptors 1/5 (mGluR1/5), D1/5 dopamine receptors, 5-HT7R serotonin receptors] and (2) downstream signaling networks [including calmodulin-dependent protein kinase II (CaMKII), protein kinase A (PKA), protein kinase C (PKC), and inositol trisphosphate receptors (IP_3Rs)]. (3) This results in activation of extracellular proteinases [tumor necrosis factor-α-converting enzyme (TACE), disintegrin and metalloprotease with thrombospondin motifs 4/5 (ADAMTS4/5), matrix metalloproteinase 9 (MMP9)], which (4) may process ECM molecules (lecticans) or (5) ECM receptors (NPR, CD44), enabling synaptic modifications (not shown) as well as (6) signaling back through modulation of GPCRs (group III mGluR) and (7) additional intracellular signaling events. (8) Inactivity increases cell surface expression and signaling through major extracellular matrix (ECM) receptors, β3 integrins. (9) Activity stimulates secretion of Narp and its coaggregation with GluAs on interneurons. (10) Intracellular signaling cascades are converging on regulation of trafficking of GluAs [homeostatic synaptic plasticity (HSP)] or voltage- and Ca^{2+}-gated ion channels (HIP).

network hyperactivity would favor subsequent induction of LTP because of the reduced initial synaptic strength. Concomitant homeostatic upregulation of HCN activity counteracts, however, the increased propensity of CA1 excitatory synapses to undergo LTP (Gasselin et al., 2015).

Similarly, downregulation of chondroitin sulfate-rich ECM increases signaling through β1 integrins, which may upregulate expression of GluN2B subunits of NMDARs (Schweitzer et al., 2017) and hence activates metaplastic mechanisms, which will promote synaptic plasticity (Song et al., 2019). These changes are counteracted by modulation of intrinsic excitability through activation of SK channels, which inhibits induction of LTP by theta-burst stimulation because of increased afterburst-hyperpolarization (Song et al., 2019).

Metaplasticity may occur also at the network level. For example K^+ channels of the K_V1 subfamily are enriched in the distal part of the AIS where they colocalize with scaffold proteins (PSD-93) and CAMs (contactin-associated protein-like 2, axonal glycoprotein TAG-1, and disintegrin and metalloproteinase domain-containing protein 22) (Leterrier, 2018). Although it is unclear whether these proteins are important for localizing K_V1 channels at the AIS, high-frequency stimulation of the Schaffer collaterals has been shown to downregulate K_V1 channel activity in hippocampal parvalbumin interneurons via activation of mGluR5. This enhances feed-forward inhibition mediated by parvalbumin interneurons, thus balancing increased synaptic and intrinsic excitation in CA1 pyramidal neurons (Campanac et al., 2013).

Another known ECM-dependent metaplastic mechanism is activated by deficiency in the ECM glycoprotein tenascin-R, which leads to upregulation of excitatory transmission to CA1 pyramidal neurons and reduction in perisomatic inhibition in the CA1 region through activation of postsynaptic metabotropic $GABA_B$ receptors. This mechanism impairs TBS-LTP and results in a 10-mV metaplastic shift in the depolarization threshold necessary to induce LTP by low-frequency stimulation (Bukalo et al., 2007). In summary, downregulation of ECM may activate homeostatic non-Hebbian plasticity (via modulation of excitability) in parallel with metaplasticity (i.e., changes in rules of Hebbian plasticity) and call for careful dissection of their interplay in the context of neurological diseases.

CONCLUDING REMARKS

We have highlighted emerging evidence suggesting a synergistic interplay between metabotropic receptors and ECM in regulating homeostatic plasticity. Activation of metabotropic receptors for glutamate, DA, and serotonin can initiate intracellular signaling pathways through tyrosine and serine kinases that culminate in the proteolytic cleavage of ECM molecules and ECM receptors. This structural remodeling of the extracellular environment provides either permissive or instructive conditions for HSP and HIP by regulating trafficking of synaptic and extrasynaptic ion channels, respectively. Furthermore, ECM proteins can also affect directly localization and signaling of metabotropic receptors. Although the experimental evidence is still scant, we propose that the superimposition of these reciprocal signaling pathways between intracellular and extracellular environments provides a robust and dynamic regulatory system for multiple forms of homeostatic plasticity (**Figure 2**).

AUTHOR CONTRIBUTIONS

LC, CV, and AD wrote the manuscript and designed figures.

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Mechanisms of Homeostatic Synaptic Plasticity *in vivo*

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Synapses undergo rapid activity-dependent plasticity to store information, which when left uncompensated can lead to destabilization of neural function. It has been well documented that homeostatic changes, which operate at a slower time scale, are required to maintain stability of neural networks. While there are many mechanisms that can endow homeostatic control, sliding threshold and synaptic scaling are unique in that they operate by providing homeostatic control of synaptic strength. The former mechanism operates by adjusting the threshold for synaptic plasticity, while the latter mechanism directly alters the gain of synapses. Both modes of homeostatic synaptic plasticity have been studied across various preparations from reduced *in vitro* systems, such as neuronal cultures, to *in vivo* intact circuitry. While most of the cellular and molecular mechanisms of homeostatic synaptic plasticity have been worked out using reduced preparations, there are unique challenges present in intact circuitry *in vivo*, which deserve further consideration. For example, in an intact circuit, neurons receive distinct set of inputs across their dendritic tree which carry unique information. Homeostatic synaptic plasticity *in vivo* needs to operate without compromising processing of these distinct set of inputs to preserve information processing while maintaining network stability. In this mini review, we will summarize unique features of *in vivo* homeostatic synaptic plasticity, and discuss how sliding threshold and synaptic scaling may act across different activity regimes to provide homeostasis.

Keywords: sliding threshold, metaplasticity, BCM theory, synaptic scaling, cortical plasticity, homeostasis, hebbian plasticity

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INTRODUCTION

A major challenge faced by neural circuits is to maintain proper neural processing while enabling effective information storage mediated by activity-dependent synaptic plasticity. This is not trivial, because plasticity of synaptic connections innately alters the flow of information between neurons. Furthermore, activity-dependent synaptic plasticity, namely long-term potentiation (LTP) and long-term depression (LTD), creates positive feedback which when uncompensated lead to network instability. In this mini review, we will compare two models of homeostatic synaptic plasticity, sliding threshold and synaptic scaling (**Figure 1**), and present emerging ideas as to how these two different models may interact to provide network stability *in vivo* (**Figure 2**).

Earlier studies on neural networks encountered difficulty in maintaining network function when solely engaging Hebbian synaptic plasticity for learning algorithms (discussed in Cooper and Bear, 2012). In one successful theory that allowed network stability developed by Leon Cooper's group, the threshold for synaptic plasticity is controlled by integrated past neuronal activity

(Bienenstock et al., 1982; Bear et al., 1987; Cooper and Bear, 2012). This theory termed the "sliding threshold" or "BCM model" not only explained development of neural feature selectivity and *in vivo* visual cortex plasticity, but it also made specific predictions that were experimentally verified subsequently (Bienenstock et al., 1982; Bear et al., 1987; Cooper and Bear, 2012). The key feature of this model is that the induction threshold for LTP and LTD is determined by past neural activity (**Figures 1A,B**). Specifically, a period of high activity increases the threshold for LTP induction, which meant most activity would fall below the synaptic modification threshold resulting in LTD. In theory, net LTD in the synaptic population should reduce neural activity even when other factors (e.g., inhibition and excitability) are unchanged. Prolonged low activity decreases the synaptic modification threshold to promote LTP across synapses. Experimental support for the sliding threshold model comes primarily from studies in sensory cortices, where sensory deprivation alters the synaptic modification threshold to favor LTP (Kirkwood et al., 1996; Hardingham et al., 2008; Guo et al., 2012).

Synaptic scaling is another popular model that provides homeostasis by adjusting the synaptic gain. While the sliding threshold model was initially proposed to explain the development of neural response selectivity and experience-dependent cortical plasticity, the premise of synaptic scaling was to explain stability of network activity propagation and firing rate homeostasis (Turrigiano and Nelson, 2004). In brief, prolonged inactivity leads to upscaling of excitatory synapses, while prolonged increase in activity downscales them to maintain overall average firing rate. Initial experimental support for synaptic scaling has come from *in vitro* neuronal culture models where activity was manipulated globally using pharmacological methods. Global inhibition of neural firing by application of tetrodotoxin (TTX) scales up excitatory synapses, while increasing neural activity by pharmacologically blocking inhibition scales down the strength of synapses (O'Brien et al., 1998; Turrigiano et al., 1998).

While both sliding threshold and synaptic scaling can provide similar homeostatic control by regulating synaptic strength, they differ in one key element. Sliding threshold model operates by altering the induction threshold for LTP/LTD, hence by nature requires neural activity to manifest the synaptic changes. Therefore, even if the synaptic modification threshold has changed based on integrated past activity, if there is insufficient neural activity through any of the synapses, there will be no change in synaptic gain. In contrast, synaptic scaling can occur without neural activity. Indeed, blocking all activity with TTX scales up excitatory synapses (O'Brien et al., 1998; Turrigiano et al., 1998). In addition, sliding threshold model posits that homeostatic control of synaptic strength will be input-specific even if the threshold is modified globally across the cell. This is because synapses that receive activity that falls below the synaptic modification threshold will undergo LTD, while those receiving activity surpassing the threshold will express LTP (Cooper and Bear, 2012). This is different from synaptic scaling where most synapses will show the same polarity of change in synaptic gain, unless the scale of operation is local as has been shown in some experimental preparations (reviewed in Turrigiano, 2008).

In the following sections, we will discuss evidence from *in vivo* preparations as to how each homeostatic synaptic plasticity model could operate, and provide evidence supporting a novel view that these two forms of homeostatic synaptic plasticity models likely operate under different activity regimes.

DEMONSTRATION OF HOMEOSTATIC SYNAPTIC PLASTICITY *IN VIVO*

Experience-dependent homeostatic synaptic plasticity has been demonstrated in various *in vivo* preparations (Whitt et al., 2014). The first experimental evidence came from studies on metaplasticity showing that prolonged visual deprivation alters the induction threshold for LTP/LTD (Kirkwood et al., 1995, 1996). Dark-rearing, expected to reduce the overall activity in visual cortex, decreased the induction threshold for LTP as predicted from the model (**Figure 1A**). Subsequent studies showed that the reduced LTP threshold resulted from an increased proportion of GluN2B-containing NMDARs at synapses (Quinlan et al., 1999; Philpot et al., 2001, 2003). GluN2B subunits have a longer current duration than GluN2A (Rumbaugh and Vicini, 1999), hence ideally suited to reduce the induction threshold for LTP. The opposite is also the case: increasing sensory experience reduces the proportion of synaptic GluN2B shifting the modification threshold to favor the induction of LTD (Quinlan et al., 1999). In parallel to sliding the induction threshold for synaptic modification, a later study demonstrated that metaplasticity can also manifest by alterations in the expression mechanisms of LTP/LTD (Huang et al., 2012). In particular, Huang et al. (2012) demonstrated that neuromodulators coupled to Gs-proteins are critical for LTP and will shift the synaptic modification function to produce an LTP-only state, while Gq-coupled neuromodulators produces an LTD-only state. This mode of metaplasticity shifts the synaptic modification curves vertically (**Figure 1B**), compared to lateral shifts produced by alterations in the induction mechanisms of LTP/LTD (**Figure 1A**). A unique aspect of this vertical shift in synaptic modification function by neuromodulators is that it puts synapses in LTP-only or LTD-only mode by changes in neuromodulatory tone coupled to internal states. Mechanistically, such vertical shift in synaptic modification function is brought about by changes in the expression mechanisms of LTP/LTD, which relates to the phosphorylation state of AMPARs (Seol et al., 2007). In particular, phosphorylation serine-845 (S845) residue on the GluA1 subunit of AMPARs is necessary for both LTP promoted by Gs-coupled neuromodulators and LTD promoted by Gq-coupled neuromodulators, while GluA1 serine-831 (S831) is necessary only for Gq-coupled neuromodulator induced LTD (Seol et al., 2007).

Visual cortex has also been a model used to demonstrate synaptic scaling *in vivo*. For example, visual deprivation in the forms of intraocular injection of tetrodotoxin (TTX) (Desai et al., 2002), dark exposure (Goel et al., 2006, 2011; Goel and Lee, 2007;

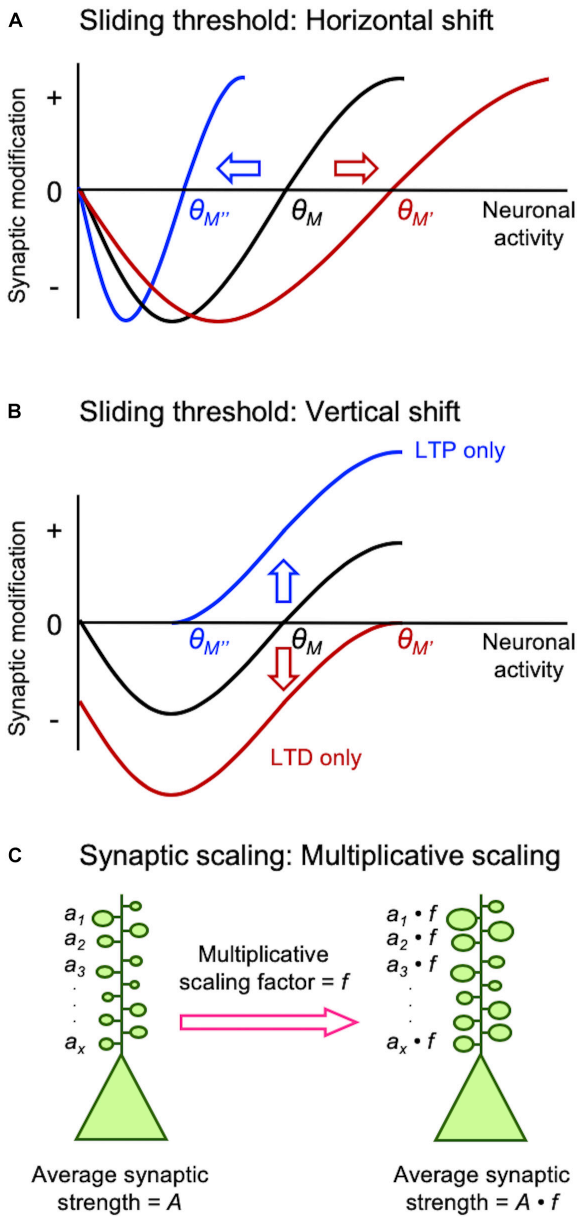

FIGURE 1 | Different models of homeostatic synaptic plasticity comparison of sliding threshold model **(A,B)** and synaptic scaling **(C)**. Sliding threshold model posits that the synaptic modification threshold (θ_M) changes as a function of past activity of a neuron. When integrated past activity is high θ_M slides up to a higher value ($\theta_{M'}$) promoting LTD, while with lower overall activity θ_M slides down to a lower value ($\theta_{M''}$) to preferential induce LTP. Expression of LTP or LTD as a consequence of sliding θ_M acts to provide homeostasis of the average neural activity. θ_M can slide via a horizontal shift **(A)**, which is implemented by altering the induction mechanisms of LTP/LTD such as regulation of GluN2B-containing NMDARs. θ_M can also slide by a vertical shift **(B)**, which is mediated by changes in the expression mechanisms of LTP/LTD such as alteration in AMPAR phosphorylation state. Synaptic scaling was initially reported to occur globally across all synapses. A key feature that allows preservation of information stored at individual synapses despite global adjustment of synaptic weights is via multiplicative scaling **(C)**. Individual synaptic weights ($a_1 \ldots a_x$) are multiplied by a same scaling factor (f), which is greater than 1 for adapting to inactivity and less than 1 for adaptation to increased activity.

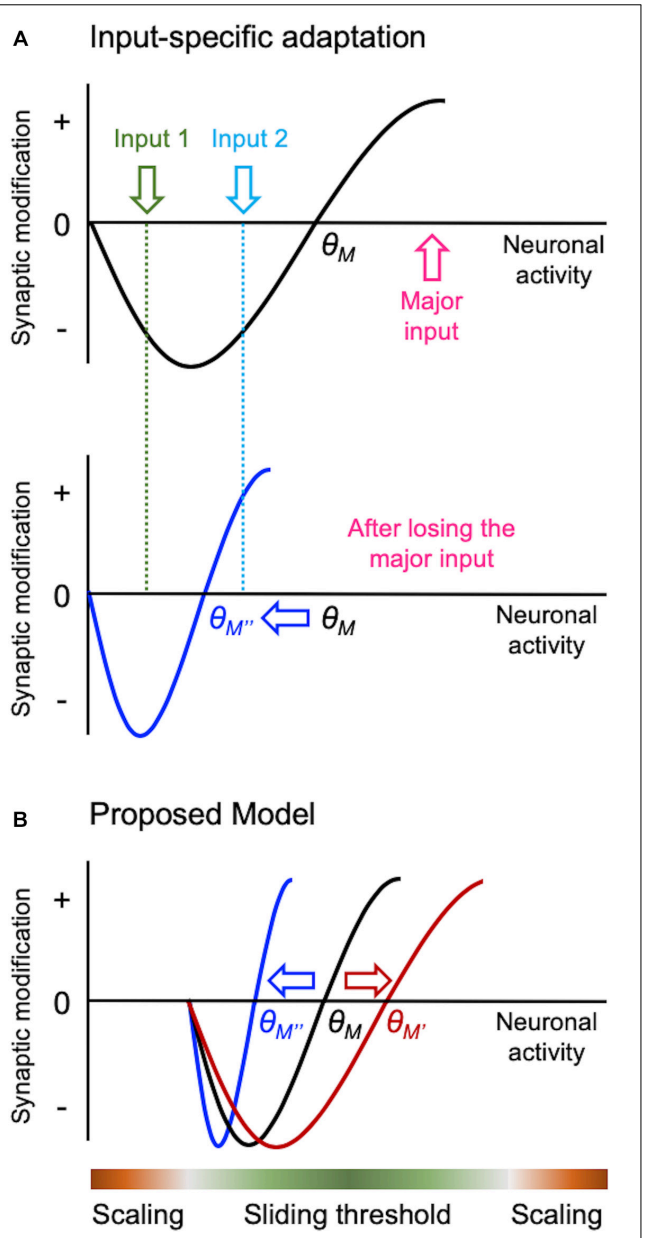

FIGURE 2 | Input-specific homeostatic synaptic plasticity and distinct activity regime. There are specific considerations needed when implementing homeostatic regulation in intact circuits *in vivo,* such as a need to provide homeostasis in an input-specific manner. Sliding threshold model can easily accomplish input-specificity as depicted in panel **(A)**. When overall activity of a neuron is reduced, such as due to loss of its major input, θ_M slides down. This causes previously weak Input 2 to cross the LTP threshold for synaptic potentiation, but leaves the less active input (Input 1) in the LTD range. Such input-specific adaptation allows the neuron to dynamically update its synaptic weights to process the most active input(s) in the context of its overall activity. We propose that sliding threshold and synaptic scaling operate across different activity regimes *in vivo* as shown in panel **(B)**. Based on the advantage sliding threshold endows intact neural networks, such as always adapting to the most relevant inputs as shown in panel **(A)**, we surmise that this is the dominant mode of homeostatic adaptation within most physiological range of activity. However, sliding threshold is less likely to be effect at providing homeostasis at extreme ranges of activity. For instance,

(Continued)

FIGURE 2 | Continued
when activity levels are too low, even if the θ_M slides, there will be insufficient activity to activate NMDARs to drive potentiation of synapses. We suggest that NMDAR-independent synaptic scaling will be more effective at providing homeostatic adaptation with inactivity. At the other extreme, synaptic scaling will be much more effective at dampening overactive circuits, because it can globally reduce the strength of synapses.

Gao et al., 2010; He et al., 2012; Petrus and Lee, 2014), dark-rearing (Goel et al., 2006), enucleation (He et al., 2012; Barnes et al., 2017), or retinal lesions (Keck et al., 2013) scales up mEPSCs. Interestingly, in V1 upscaling of mEPSCs has layer specific sequential critical periods, where layer 4 (L4) ends by postnatal day 21(P21) (Desai et al., 2002) while in layers 2/3 (L2/3) it starts by P21 and persist through adulthood (Goel and Lee, 2007). The rates of scaling up and down are asymmetric. It takes at least 2 days of darkness to upscale mEPSCs (Goel and Lee, 2007), but only 2 h of light re-exposure to fully reverse it (Gao et al., 2010), suggesting different temporal integration for each process. Experience-dependent synaptic scaling has been reported in other sensory cortices besides V1: in L2/3 of auditory cortex after sensorineural hearing loss (Kotak et al., 2005) or conductive hearing loss (Teichert et al., 2017), in L4 of barrel cortex after afferent nerve (i.e., infraorbital nerve) transection (Yu et al., 2012), but not in L2/3 of barrel cortex after whisker plucking (Bender et al., 2006; He et al., 2012; Li et al., 2014) (but see Glazewski et al., 2017). This intriguing absence of synaptic scaling with whisker plucking will be discussed in section "Specific Challenges Of Homeostatic Synaptic Plasticity *in vivo*."

Mechanistically, scaling up and down are not the reverse of each other, but rely on distinct molecular signaling. In V1, upscaling of mEPSCs after DE correlates with phosphorylation of GluA1 on S845, synaptic appearance of Ca^{2+}-permeable AMPARs (Goel et al., 2006), and mGluR1 (Chokshi et al., 2019), while downscaling is dependent on Arc (Gao et al., 2010), mGluR5, and Homer1a (Chokshi et al., 2019). Although GluA1-S845 is necessary for upscaling, it alone is not sufficient to recapitulate multiplicative scaling (Goel et al., 2011). Multiplicative change is a key feature of synaptic scaling (**Figure 1C**), because it preserves information stored as different weights across synapses in a neuron (Turrigiano et al., 1998). However, multiplicative scaling is only observed early in development (P21 to ∼P35) in V1 (Goel and Lee, 2007). We interpreted this to suggest that synaptic scaling in adults is not global, but limited to a subset of synapses. Consistent with this interpretation, we reported that DE-induced upscaling of mEPSCs reflects potentiation of lateral intracortical (IC) synapses, but feedforward (FF) synapses from L4 to L2/3 are immune to this type of plasticity (Petrus et al., 2015). Similarly, downscaling of mEPSCs with visual experience is also limited to IC synapses (Chokshi et al., 2019). Such input-specific synaptic scaling is observed in L5 of V1 at the level of dendritic spine plasticity. It was reported that visual deprivation via enucleation leads to enlargement of dendritic spines on L5 neurons, which was specific to dendritic branches with recent spine loss (Barnes et al., 2017). Based on these new observations showing that sensory experience-dependent homeostatic plasticity of mEPSCs is input-specific and also other recent evidence discussed below, we propose that the apparent synaptic scaling induced *in vivo* with sensory manipulations is actually a manifestation of sliding threshold metaplasticity see section "Different Activity Regime May Recruit Distinct Homeostatic Synaptic Plasticity *In vivo*."

SPECIFIC CHALLENGES OF HOMEOSTATIC SYNAPTIC PLASTICITY *IN VIVO*

One of the challenges of homeostatic plasticity operating *in vivo* is that not all inputs are identical. Cortical neurons receive diverse set of inputs from multiple sources. For example, V1 not only receives inputs from the primary visual thalamus (dLGN), but also from other sensory areas (Lakatos et al., 2007; Iurilli et al., 2012; Yoshitake et al., 2013; Ibrahim et al., 2016), subcortical areas (Roth et al., 2016), higher visual areas (Coogan and Burkhalter, 1993; Dong et al., 2004; Ji et al., 2015; Marques et al., 2018), and other cortical areas (Wall et al., 2016). Input diversity is not a particular property of V1, but rather a general property of highly interconnected cortical networks. It is inconceivable then that all of the inputs are equivalent and share the same levels of input activity. Therefore, homeostatic synaptic plasticity needs to occur in a way to preserve information storage and processing capacity of a diverse set of networks in which a particular neuron participates in. It was proposed based on computational modeling that input-specific homeostatic plasticity is much better suited to improve information processing than global synaptic scaling (Barnes et al., 2017) (for further discussions see Keck et al., 2017). In this particular study, the unit of homeostatic control was proposed to be a dendritic branch. There are several observations that similar inputs tend to cluster on the same dendritic branch (Wilson et al., 2016; Iacaruso et al., 2017), thus branch-specific homeostatic adaptation would allow functional input-specific control that is independent from each other.

Another unique challenge to study *in vivo* homeostatic plasticity is that not all sensory manipulations lead to the same changes. As mentioned above, in the case of visual deprivation, majority of the paradigms ranging from intraocular TTX injection, dark-rearing, dark-exposure, enucleation, and retinal lesions scales up mEPSCs in V1 (Desai et al., 2002; Goel et al., 2006; Goel and Lee, 2007; He et al., 2012; Keck et al., 2013; Barnes et al., 2017). However, lid suture typically do not (Maffei and Turrigiano, 2008; He et al., 2012; Bridi et al., 2018) (but see Hengen et al., 2013). Similarly, in the barrel cortex afferent nerve transection upregulates mEPSCs (Yu et al., 2012; Chung et al., 2017), but not whisker deprivation (Bender et al., 2006; He et al., 2012; Li et al., 2014); but see Glazewski et al. (2017). Differences in outcome may stem from the degree of activity changes associated with various sensory manipulations. In the visual deprivation cases, dark-rearing or dark-exposure removes vision, but leaves spontaneous activity in the retina and through the visual pathway. Recently, we reported that dark-exposure for a few days lead to increase in spontaneous firing of V1 neurons

(Bridi et al., 2018). Intraocular TTX injection and enucleation removes vision and spontaneous activity in the retina, but it has been noted that dLGN neurons undergo oscillatory activity (Linden et al., 2009). Lid suture is a much milder form of deprivation where form vision is largely lost, but vision is not totally abolished. Visual stimulation seen through the closed eyelids can elicit small but measurable visually evoked potentials (VEPs) in V1 (Blais et al., 2008). As exemplified, the level of sensory deprivation and the consequent changes in neural activity through the sensory pathway is not identical across different paradigms. This is not likely just limited to the visual system, but it extends to other sensory cortices. For example, the reason that whisker deprivation in most cases fails to induce changes in mEPSCs in barrel cortex L2/3 (Bender et al., 2006; He et al., 2012; Li et al., 2014) may be because it is similar to lid suture where afferent activity is not completely abolished. In any case, study of homeostatic plasticity *in vivo* will need to be interpreted in the framework of the specific type of manipulation done, which adds complication compared to pharmacological manipulation of activity that can be achieved *in vitro*.

Further complications when studying intact cortical circuits is that one needs to consider the specific cell-type and lamina that is being investigated. One reason is that different laminae exhibit distinct critical period for plasticity with L4 typically showing early plasticity followed by opening of plasticity in L2/3 (Desai et al., 2002; Goel and Lee, 2007; Jiang et al., 2007). Also the means in which different laminar neurons adapt to the same types of sensory manipulations are quite distinct (reviewed in Whitt et al., 2014; also see Glazewski et al., 2017). Even within the same layer, cell type also seems to matter. For example, in L5 of barrel cortex, there is distinct plasticity triggered by changes in sensory experience based on specific cell-types (Greenhill et al., 2015; Glazewski et al., 2017). Ultimately, there will be differences in input activity based on the different functional circuit in which a particular neuron is part of. Hence, it is not surprising that different neurons would respond differently to a particular *in vivo* manipulation.

DIFFERENT ACTIVITY REGIME MAY RECRUIT DISTINCT HOMEOSTATIC SYNAPTIC PLASTICITY *IN VIVO*

There is emerging evidence that different activity regimes may recruit distinct modes of homeostatic adaptation *in vivo* (**Figure 2B**). Bridi et al. reported that visual deprivation leads to metaplasticity mode of homeostatic adaptation in V1, but silencing cortical activity more by pharmacologically increasing tonic inhibition produces synaptic scaling-like adaptation (Bridi et al., 2018). Of interest is that visual deprivation-induced metaplasticity is likely driven by increased spontaneous activity acting on GluN2B-containing NMDARs. This counters the conventional notion that sensory deprivation leads to loss of activity in the corresponding sensory cortex, and that inactivity is driving homeostatic adaptation. This work suggests that sensory deprivation-induced homeostatic plasticity requires activity, for instance, in the form of elevated spontaneous activity. We also recently reported that dark-exposure induced upscaling of mEPSCs in V1 L2/3 is dependent on NMDAR activity (Rodriguez et al., 2019), which further corroborates the involvement of sliding threshold that acts on NMDAR-dependent LTP/LTD processes. Our current working model is that sensory deprivation-induced reduction in synaptic modification threshold coupled with increased spontaneous activity potentiates synapses to mediate homeostatic increase in excitatory synaptic gain. Increased spontaneous activity has been reported in A1 with auditory deprivation (Kotak et al., 2005), and infraorbital nerve transection that potentiates synapses in barrel cortex also increases GluN2B-containing NMDARs (Chung et al., 2017). These findings suggest that similar mechanism may operate across sensory cortices.

Sliding threshold mediated homeostatic adaptation has an advantage that it can easily implement input-specificity (**Figure 2A**). Inputs that exhibit activity above the threshold will produce potentiation, those falling below will depress, and inputs with minimal activity or activity at the threshold will not change. Such input-specific homeostatic adaptation has one advantage in that it will allow the circuit to preferentially process currently active inputs despite overall activity changes. Therefore, the cortical networks can be dynamically reconfigured for processing the most relevant information in the context of overall activity in the circuit. It is of interest to note that input-specific homeostatic plasticity is more prevalent in mature cortex (Goel and Lee, 2007; Ranson et al., 2012; Petrus et al., 2015; Barnes et al., 2017; Chokshi et al., 2019).

While sliding threshold provides homeostasis with sensory manipulation paradigms, synaptic scaling seems to also be present *in vivo* but at extreme activity ranges (**Figure 2B**). For example, reducing cortical activity by pharmacologically increasing tonic inhibition leads to upscaling of mEPSCs, which is not dependent on NMDARs (Bridi et al., 2018). We surmise that synaptic scaling may also operate when neural activity is increased to an extreme level. The rationale is that under either extreme activity regimes sliding threshold may not be effective. For example, under extremely low activity even if the synaptic modification threshold slides down, there may not be sufficient level of activity to drive LTP. Therefore, NMDAR-independent plasticity, such as synaptic scaling, may be better suited for synaptic adjustments under this condition. Similarly, when there is extremely high neural activity across all inputs, as would occur during seizures, having input-independent global synaptic scaling is likely a more efficient way to dampen activity.

CONCLUSION

We summarized the specific challenges met when homeostatic plasticity operates in intact circuits *in vivo* with diverse sets of inputs. We propose that sliding threshold operates across activity ranges that can recruit NMDAR-dependent input-specific synaptic plasticity to maintain optimal processing of most relevant information despite overall changes in activity, while synaptic scaling may operate at extreme activity ranges to act as a failsafe.

AUTHOR CONTRIBUTIONS

Both authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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miRNA-Dependent Control of Homeostatic Plasticity in Neurons

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Homeostatic plasticity is a form of plasticity in which neurons compensate for changes in neuronal activity through the control of key physiological parameters such as the number and the strength of their synaptic inputs and intrinsic excitability. Recent studies revealed that miRNAs, which are small non-coding RNAs repressing mRNA translation, participate in this process by controlling the translation of multiple effectors such as glutamate transporters, receptors, signaling molecules and voltage-gated ion channels. In this review, we present and discuss the role of miRNAs in both cell-wide and compartmentalized forms of homeostatic plasticity as well as their implication in pathological processes associated with homeostatic failure.

Keywords: homeostatic plasticity, miRNA–microRNA, synaptic scaling, protein translation, membrane excitability, synaptic strength, synaptic plasticity

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INTRODUCTION

Neurons employ a variety of homeostatic mechanisms to maintain network activity within physiological ranges in response to a wide range of remodeling events. These include for instance the assembly of synaptic circuits during development, learning and memory, or the pathological loss of synapses associated with neurodegenerative disorders (Hengen et al., 2013; Keck et al., 2013; Vitureira and Goda, 2013; Fernandes and Carvalho, 2016; Turrigiano, 2017). Depending on the situation, such homeostatic mechanisms may involve different signaling pathways, act on various physiological parameters, and operate on multiple time and space scales (reviewed in Turrigiano, 2012; Vitureira et al., 2012; Fernandes and Carvalho, 2016). It is therefore not surprising that the failure of neuronal homeostasis can impact physiological processes such as memory consolidation and synaptic circuit refinement (Mrsic-Flogel et al., 2007; Hengen et al., 2016; Diering et al., 2017), can contribute to epilepsy (Swann and Rho, 2014) and to various neurological disorders (Ramocki and Zoghbi, 2008; Dickman and Davis, 2009; Wondolowski and Dickman, 2013; Nelson and Valakh, 2015; Penn et al., 2017).

One important feature shared among the multiple forms of homeostatic plasticity is that they are slow as compared to Hebbian forms of plasticity, i.e., long-term potentiation (LTP) or depression (LTD), in which synaptic strengths are rapidly and durably potentiated or depressed, respectively. Homeostatic plasticity usually develops over the course of several hours, and up to several days, and relies on the synthesis of new proteins which regulate key physiological parameters (Turrigiano, 2012; Fernandes and Carvalho, 2016). Proteins as diverse as glutamate receptors (e.g., AMPARs), scaffolding proteins (e.g., PSD-95, PICK1), voltage-gated ion channels (e.g., P/Q-type

calcium channels), kinases (e.g., CAMKIIβ, PKA), cell-adhesion molecules (e.g., β3-integrins) or soluble factors (e.g., TNFα, retinoic acid, BDNF) contribute to homeostatic plasticity through the regulation of synaptic efficacy, synapse number, and/or membrane excitability (reviewed in Turrigiano, 2012; Fernandes and Carvalho, 2016). So far, several studies have uncovered the role of activity-dependent mRNA transcription of immediate early genes like Plk2, Homer1a, Arc, and Narp (Shepherd et al., 2006; Seeburg et al., 2008; Chang et al., 2010; Gao et al., 2010; Diering et al., 2017) and the contribution of transcription regulators such as MSK1, MeCP2, and CaMKIV (Ibata et al., 2008; Blackman et al., 2012; Correa et al., 2012; Qiu et al., 2012). In contrast to transcriptional studies, a potential contribution of mechanisms regulating *de novo* protein synthesis at the post-transcriptional level such as mRNA translation and/or stability is just emerging (Fernandez-Moya et al., 2014; Kosik, 2016).

Among the actors that may be involved in these processes, microRNAs (miRNAs) appear as important regulators of homeostatic plasticity in the nervous system. These small non-coding RNAs are highly enriched in the brain where they regulate a very large number of genes and shape transcriptomic diversity across regions (Filipowicz et al., 2008; Friedman et al., 2008; Soula et al., 2018). miRNAs are first synthesized in the nucleus then loaded in the RNA induced silencing complex (RISC), where they hybridize to the 3′ UTR of target mRNAs and inhibit protein synthesis through translational repression or destabilization of the transcript (**Figure 1**). The sequence involved in miRNA–mRNA interaction is called the "seed" region and is composed of the nucleotides 2–8 of the 5′ region of the miRNA (Bartel, 2009). Due to the small size of the "seed" region and the length of 3′ UTRs, the translation of a given mRNA is often under the control of multiple miRNAs while individual miRNAs can regulate the expression of dozens, if not 100s, of genes (Friedman et al., 2008). Loss of function approaches targeting individual miRNAs or their maturation through the endoribonuclease Dicer (Giraldez, 2005; Kim et al., 2007; Cuellar et al., 2008; Störchel et al., 2015; Fiorenza et al., 2016) have unveiled a contribution of the miRNA system in most aspects of neuronal development and plasticity, including neuronal differentiation and survival, neurite growth, synapse development, and plasticity (Kosik, 2006; Fineberg et al., 2009; Follert et al., 2014; Hu and Li, 2017; Tien and Kerschensteiner, 2018). In comparison with the regulation of transcription, which is spatially restricted to the nucleus, miRNAs provide an additional layer of regulations to finely tune in time and space protein synthesis in remote subcellular compartments such as synapses, and help cells adapt to their complex environment (**Figure 1**).

In this review, we present recent advances showing the contribution of several miRNAs in both cell-wide and compartmentalized forms of homeostatic plasticity through the regulation of the translation of multiple effectors (**Figure 1** and **Table 1**). We first focus on homeostatic plasticity mechanisms that are regulated by miRNAs at the pre and post-synaptic levels, then discuss the impact of miRNAs on experience-dependent homeostatic synaptic plasticity (HSP) and neuronal excitability. Finally, we discuss several important questions that remain to be addressed, including the local versus global miRNA regulation and the implication of miRNAs in neuronal diseases.

miRNA-DEPENDENT CONTROL OF POST-SYNAPTIC FUNCTION DURING HOMEOSTATIC SYNAPTIC PLASTICITY

One parameter that is commonly regulated to maintain synaptic homeostasis is the abundance of post-synaptic receptors. At excitatory synapses, the accumulation or depletion of synaptic AMPA-type glutamate receptors (AMPARs) has been well-characterized, mostly in primary neuronal cultures, following prolonged deprivation or elevation of neuronal activity, respectively. Depending on how neuronal activity is altered, this plasticity can be cell-wide or synapse-specific, and can engage different signaling pathways and combinations of AMPAR subunits (Vitureira and Goda, 2013; Fernandes and Carvalho, 2016). Interestingly, global pharmacological manipulations of neuronal activity known to induce HSP (Turrigiano et al., 1998; Thiagarajan et al., 2005; Sutton et al., 2006) alter the expression of several miRNAs in primary hippocampal cultures, which likely contribute to the proteome remodeling observed upon such conditions (Schanzenbächer et al., 2016, 2018).

Homeostatic Increase of Post-synaptic Strength in Response to Activity Deprivation

In rat cultured hippocampal neurons, the blockade for $>$ 4 h of action potentials (APs) and NMDA receptors (NMDARs) with tetrodotoxin (TTX) and APV, respectively, leads to the local synthesis and synaptic insertion of AMPARs likely formed of GluA1 homomers (Sutton et al., 2006). This process is mediated by a decrease of miR-92a targeting the AMPAR subunit GluA1 in dendrites (Letellier et al., 2014). As a result, GluA1 translation is de-repressed and new AMPARs are targeted to synapses to support the increase in synaptic strength (Letellier et al., 2014). Importantly, this form of HSP is maintained in dendrites disconnected from the cell body (Sutton et al., 2006; Letellier et al., 2014), suggesting that transcription is not required and that the miR-92a-dependent GluA1 translation occurs locally. Intriguingly, incubating hippocampal neurons with TTX/APV for longer periods ($>$12 h) increases the expression of another miRNA, miR-124, which targets the GluA2 AMPAR subunit (Ho et al., 2014; Hou et al., 2015). While both miR-92a downregulation and miR-124 upregulation promote the expression of GluA2-lacking, calcium permeable AMPARs, the TTX/APV-induced elevation of miR-124 seems to rely on transcription-dependent mechanisms (Hou et al., 2015) and therefore may affect synaptic strengths more widely and uniformly as compared to miR-92a.

Interestingly, a 24 h activity-deprivation paradigm in cultured hippocampal neurons using non-competitive antagonists of AMPARs and NMDARs (GYKI-52466 and MK-801, respectively) does not affect miR-92a or miR-124 levels but rather downregulates miR-186-5p, a miRNA which also targets

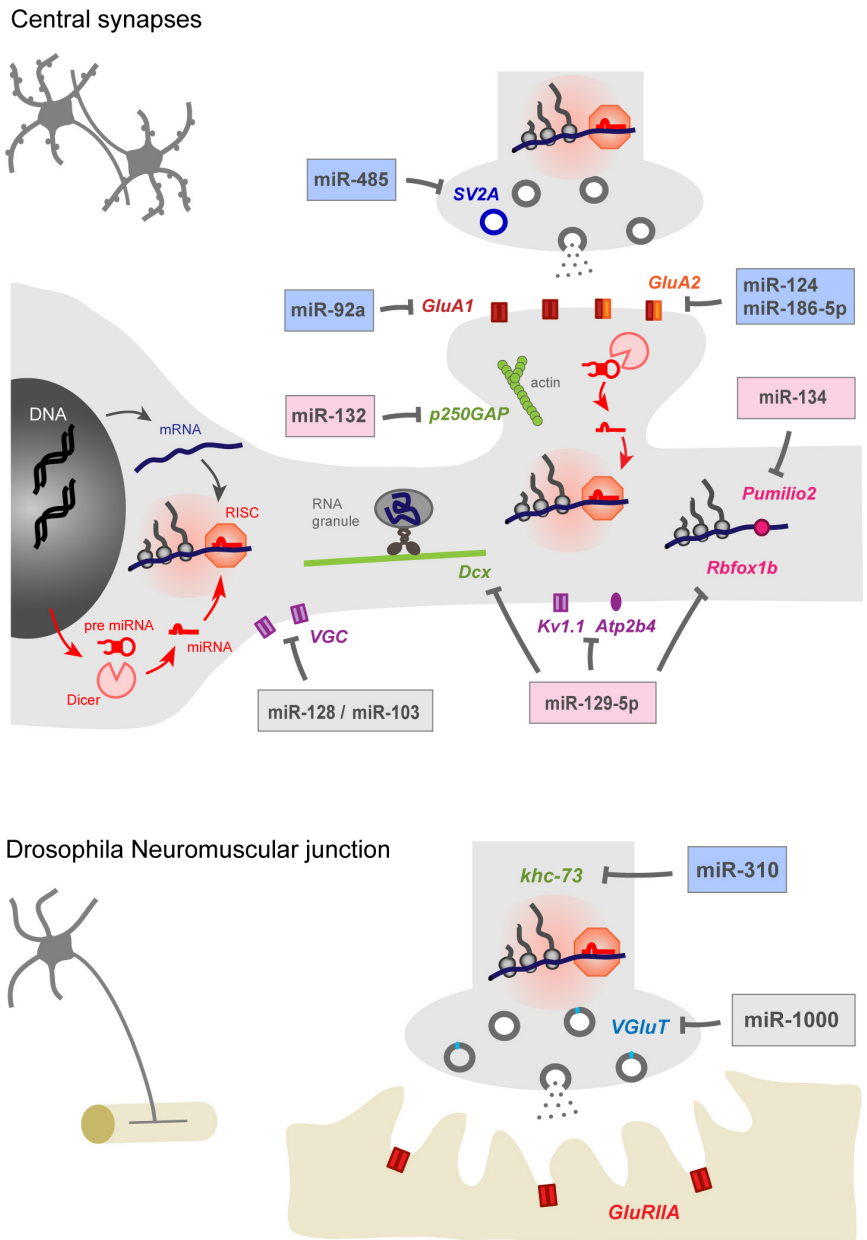

FIGURE 1 | miRNAs contribute to homeostatic plasticity by controlling multiple effectors at central synapses of rodent models **(top)** and at the drosophila neuromuscular junction **(bottom)**. Identified miRNAs target presynaptic proteins regulating neurotransmitter release, post-synaptic AMPAR subunits, cytoskeleton-related proteins, voltage gated ion channels, calcium pumps, and RNA-binding proteins. miRNAs may repress protein translation at the cell body or in pre- or post-synaptic compartments, thereby providing autonomy to subcellular compartments and regulating appropriate physiological parameters, such as pre- and post-synaptic strengths and membrane excitability. miRNAs involved in the homeostatic up- or down-regulation of neuronal activity are highlighted in blue and pink while those showing bidirectional regulations are indicated in gray. Note that the schematic for rodent central synapses summarizes collective data from several neuronal types (see **Table 1** for details).

GluA2, thereby leading to the synaptic insertion of GluA2-containing AMPARs which are not permeable to calcium (Silva et al., 2019). Finally, a 24–48 h treatment with TTX alone induces the insertion of GluA2-containing AMPARs (Sutton et al., 2006; Gainey et al., 2009), but a specific regulation of this process by miRs has not been reported yet. Together, these studies suggest that neurons engage different miR-dependent pathways depending on the activity-deprivation paradigm, to produce a selective homeostatic compensation with regards to the AMPAR subunit composition that confers specific plastic properties to synapses (Diering and Huganir, 2018). A key point will be to determine the functional significance of these multiple miRNA-dependent regulations, and whether they extend to more physiological –*in vivo*- systems and to other brain regions.

TABLE 1 | miRNAs involved in homeostatic plasticity and associated with neurological disorders.

	miRNAs involved in homeostatic plasticity	Target(s)	Homeostatic plasticity paradigm(s)	Possible associated neurological disorder(s)
Neurotransmitter release	miR-485 (Cohen et al., 2011) *Rat hippocampal cultures*	SV2A	Activity elevation (BIC + 4-AP/5 days)	Traumatic brain injury (Redell et al., 2009) Alzheimer (Lau et al., 2013)
	miR-1000 (miR-137 ?) (Verma et al., 2015) *Drosophila visual system*	VGlut	Dark rearing/constant light rearing	Rett syndrome (Szulwach et al., 2010) Alzheimer (Geekiyanage and Chan, 2011) Schizophrenia (Kwon et al., 2013) Rett syndrome (Cheng et al., 2014)
Post-synaptic receptors	miR-92a (Letellier et al., 2014) *Rat hippocampal cultures*	GluA1	Activity deprivation (TTX + AP5/4 h)	Rett syndrome (Urdinguio et al., 2010; Cheng et al., 2014) Autism spectrum disorder (Talebizadeh et al., 2008) Amyotrophic lateral sclerosis (Campos-Melo et al., 2018) Alzheimer (Patrick et al., 2017) Traumatic brain injury (Redell et al., 2009)
	miR-124 (Hou et al., 2015; Gilbert et al., 2016) *Rat hippocampal cultures*	GluA2	Activity deprivation (TTX + APV/15 h; TTX 24 h)	Huntington (Packer et al., 2008) Alzheimer (Lau et al., 2013; Gilbert et al., 2016) Epilepsy (Peng et al., 2013) Fragile-X syndrome (Xu et al., 2011) Rett syndrome (Urdinguio et al., 2010)
	miR-186-5p (Silva et al., 2019) *Rat hippocampal cultures*	GluA2	Activity deprivation (GYKI-52466 + MK-801/24 h)	Alzheimer (Kim et al., 2016) Autism spectrum disorder (Sarachana et al., 2010) Rett syndrome (Urdinguio et al., 2010; Cheng et al., 2014) 22q11.2 deletion syndrome (Stark et al., 2008)
	miR-218 (Rocchi et al., 2019) *Rat hippocampal cultures*	GluA2	Activity deprivation (TTX 48 h) Activity elevation (BIC + 4-AP 48 h)	Epilepsy (Kaalund et al., 2014) Stress-related disorders (Torres-Berrío et al., 2017) Rett syndrome (Cheng et al., 2014)
Cytoskeleton dynamics and trafficking	miR-310 (Tsurudome et al., 2010) *Drosophila NMJ*	Khc-73	GluRII mutant	
	miR-132 (Mellios et al., 2011; Tognini et al., 2011) *Mouse visual cortex*	P250GAP	Monocular deprivation	Huntington (Packer et al., 2008) Alzheimer (Lau et al., 2013; Patrick et al., 2017) Autism spectrum disorder (Abu-Elneel et al., 2008; Talebizadeh et al., 2008; Sarachana et al., 2010) Epilepsy (Peng et al., 2013) Schizophrenia (Kim et al., 2010)
RNA-binding proteins	miR-134 (Fiore et al., 2014) *Rat hippocampal cultures*	Pumilio-2	Activity elevation (PTX 48 h)	Epilepsy (Peng et al., 2013) 22q11.2 deletion syndrome (Stark et al., 2008) Rett syndrome (Urdinguio et al., 2010; Cheng et al., 2014)
	miR-129-5p (Rajman et al., 2017) *Rat hippocampal cultures*	Rbfox1 *Atp2b4* *Dcx*	Activity elevation (PTX 48 h)	Alzheimer (Lau et al., 2013; Patrick et al., 2017) Autism spectrum disorder (Abu-Elneel et al., 2008)
VGCCs	miR-103 (Favereaux et al., 2011) *Rat spinal chord neurons*	Cav1.2	Neuropathic rats	Alzheimer (Yang et al., 2018) Chronic Pain (Favereaux et al., 2011) Autism spectrum disorder (Sarachana et al., 2010) Traumatic brain injury (Redell et al., 2009) 22q11.2 deletion syndrome (Stark et al., 2008)

Homeostatic Decrease of Post-synaptic Strengths in Response to Activity Elevation

The expression of some specific miRNAs is also altered following pharmacological manipulations to elevate network activity (Fiore et al., 2014; Rajman et al., 2017; Rocchi et al., 2019), suggesting that miRNAs bi-directionally adapt synaptic strengths across dendrites depending on network activity. In cultured hippocampal neurons, miR-134 elevation induced by the chronic (>24 h) pharmacological blockade of $GABA_A$ receptors ($GABA_A$Rs) using picrotoxin (PTX) contributes to homeostatic synaptic downscaling by decreasing GluA2 surface expression and by promoting the elimination of excitatory synapses (Fiore et al., 2014). Specifically, miR-134 downregulates the RNA-binding protein Pumilio 2 which normally inhibits the polo-like kinase 2 (Plk2) pathway that promotes homeostatic downscaling through the degradation of the spine-associated protein RapGAP SPAR (SPAR) and the sequestration of the GluA2-interacting *N*-ethylmaleimide-sensitive fusion (NSF) protein (Seeburg et al., 2008; Evers et al., 2010). Curiously, other known targets of miR-134 including the protein kinase Limk1 which promotes spine development by regulating actin dynamics (Schratt et al., 2006) are not affected by the PTX treatment (Fiore et al., 2014), suggesting a selective effect. Interestingly, miR-134 is also upregulated in the temporal lobe neocortex of patients with epilepsy (Jimenez-Mateos et al., 2012). While it is currently unknown whether Pumilio 2 is downregulated in this

condition, Limk1 expression level is decreased, which could result in smaller dendritic spines to dampen hyperactivity and may represent some homeostatic adaptation (Jimenez-Mateos et al., 2012). Surprisingly, however, silencing miR-134 in mice using antagomirs suppresses seizures and has a neuroprotective action (Jimenez-Mateos et al., 2012), suggesting that abnormal increased levels of miR-134 may rather promote epilepsy. Therefore, despite the therapeutical potential of miR-134 antagomirs in the context of epilepsy, more investigations are required to understand the exact mode of action of miR-134 *in vivo*.

In another study, miR-129-5p elevation was also shown to be required for the PTX-induced downscaling of synaptic strength, by promoting the downregulation of the calcium pump Atp2b4 and the microtubule-associated protein doublecortin (Dcx) (Rajman et al., 2017). Furthermore, the authors uncover a functional interaction between miR-129-5p and the RNA binding protein Rbfox1, which normally promotes the expression of both Atp2b4 and Dcx through their 3′ UTR. Upon PTX-induced synaptic scaling, Rbfox1 expression is downregulated in a miR-129-5p manner, thereby allowing the repression of Atp2b4, Dcx and possibly other synaptic genes. However, how miR-134 and miR-129-5p work in conjunction to drive homeostatic downscaling triggered by GABAR blockade and whether other regulated miRNAs including miR-132, miR-495, miR-543-3p, or miR-218 contribute to this process (Rajman et al., 2017; Rocchi et al., 2019) remain to be investigated.

miRNA-DEPENDENT CONTROL OF PRESYNAPTIC FUNCTION DURING HOMEOSTATIC SYNAPTIC PLASTICITY

While many studies have uncovered post-synaptic mechanisms for homeostatic synaptic plasticity, there is strong evidence that neurons can also regulate the number and efficacy of presynaptic release sites to compensate for prolonged perturbations in network activity (Thiagarajan et al., 2005; Jakawich et al., 2010; Lindskog et al., 2010; Vitureira et al., 2011; Davis and Müller, 2015). Several miRNAs likely contribute to this process by targeting presynaptic proteins. For instance, in cultured hippocampal neurons, miR-485 is upregulated following the chronic elevation of neuronal activity (>5 days) using bicuculline and 4-aminopyridine (4-AP) to block $GABA_A$Rs and potassium channels, respectively (Cohen et al., 2011). miR-485 targets the synaptic vesicle protein 2A (SV2A) which is known to facilitate neurotransmitter release through an interaction with synaptotagmin (Custer, 2006; Yao et al., 2010), and which is downregulated following seizures in the hippocampus, thus possibly representing a homeostatic mechanism (van Vliet et al., 2009). Surprisingly, miR-485 expression does not downregulate presynaptic neurotransmitter release *per se* but rather decreases the number of functional synapses, as evidenced by a decreased density of PSD-95 and AMPAR clusters, suggesting a functional crosstalk between pre and post-synaptic elements. In any case, the mechanism by which miR-485 adapts the number of functional synapses in response to elevated network activity (and possibly during epilepsy) remains to be clarified, as other targets of miR-485 may also contribute to this process (Cohen et al., 2011).

One model system that has been extensively studied in the context of presynaptic homeostatic plasticity is the drosophila neuromuscular junction, where experimentally reducing the sensitivity or the expression of post-synaptic glutamate receptors is precisely balanced by an increase in glutamate release through retrograde signaling (Petersen et al., 1997; Frank et al., 2006; Frank, 2014; Davis and Müller, 2015). The miR-310-313 cluster contributes to this process most likely by targeting the kinesin family member khc-73 in motor neurons (Tsurudome et al., 2010). Specifically, overexpressing miR-310 or knocking-down khc-73 in motor neurons both inhibit the homeostatic increase in quantal content normally observed in *GluRIIA* mutants.

miR-1000 is another drosophila miRNA which modulates glutamate release by down-regulating the expression of the glutamate transporter VGlut (Verma et al., 2015). miR-1000 genetic deletion enhances VGlut expression, resulting in an excess of glutamate release through a higher number of active boutons, which are also bigger in size. Interestingly, miR-1000 expression level in the drosophila visual system is regulated in an homeostatic manner by visual input. Indeed, miR-1000 transcript levels are significantly reduced in dark-reared flies, raising the possibility that glutamate release is enhanced and compensates for reduced sensory input. In contrast, flies reared in constant light show increased miR-1000 expression compared to animals reared under a normal light-dark cycle, suggesting a reduction of glutamate release to compensate for a prolonged elevation of sensory input (Verma et al., 2015). Importantly, the failure of miR-1000-dependent regulation of glutamate release results in excitotoxicity and reduced neuron survival. While miR-1000 is not expressed in mammals, the seed-similar miRNA miR-137 is expressed in mouse hippocampal neurons and may similarly regulate VGluT2 (Verma et al., 2015) in addition to its post-synaptic target GluA1 (Olde Loohuis et al., 2015). Interestingly, miR-137 has been genetically associated with schizophrenia and miR-137 overexpression in the mouse dentate gyrus impairs presynaptic plasticity and hippocampus-dependent learning and memory through the regulation of the presynaptic proteins synaptotagmin-1, complexin-1, and NSF (Siegert et al., 2015).

miRNA-DEPENDENT CONTROL OF EXPERIENCE-DEPENDENT HOMEOSTATIC SYNAPTIC PLASTICITY *IN VIVO*

Besides compensating for global perturbations of network activity, whether induced pharmacologically or genetically (see above), there is evidence that HSP also contributes to experience-dependent plasticity and refinement of developing synaptic circuits. In such situations, the strengthening of active inputs is compensated by the weakening of less active inputs on the target cell, presumably through competition-based mechanisms; this eventually leads to the selective stabilization of specific

inputs at the expense of others. This activity-dependent process has been extensively studied in the mammalian visual cortex where a population of neurons respond to the two eyes. Occluding the vision of one eye during a critical developmental period (monocular deprivation paradigm), produces a loss of responsiveness of binocular neurons to the deprived eye which is precisely balanced by a corresponding homeostatic increase in response to the undeprived eye (ocular dominance shift), thus preserving the net visual drive for each neuron (Mrsic-Flogel et al., 2007; Kaneko et al., 2008; Ranson et al., 2012; Kaneko and Stryker, 2017).

Interestingly, the expression of some specific miRNAs is altered following sensory deprivation in the visual cortex, and may contribute to the homeostatic component of the ocular dominance shift. Among them, miR-132, is decreased after monocular deprivation or dark rearing (Mellios et al., 2011; Tognini et al., 2011). Inhibiting miR-132 through the injection of a miRNA-sponge-expressing lentivirus (Mellios et al., 2011) or counteracting miR-132 reduction by infusing a miR-132 mimic (Tognini et al., 2011) both prevent the ocular dominance plasticity shift induced by monocular sensory deprivation, suggesting that miR-132 drop is necessary to both weaken the deprived visual input and strengthen the undeprived input. At the cellular level, such a homeostatic balance between active and inactive inputs may involve the de-repression of the miR-132 target p250GAP, a Rho family GTPase that regulates spine morphology and remodeling through Rac1 inhibition (Wayman et al., 2008; Edbauer et al., 2010; Impey et al., 2010; Remenyi et al., 2013) and which has been implicated in epileptogenesis process (Yuan et al., 2016). In one possible mechanism, sensory-deprived synaptic inputs could depress and shrink through the GTPase p250GAP/Rac1 pathway while more active synapses would get strengthened and grow in size. Importantly, the differential regulation of active versus inactive synapses in the same post-synaptic cell suggests the existence of local signaling within dendrites (Oh et al., 2015; El-Boustani et al., 2018; Letellier et al., 2019) to which miRNAs might contribute.

miRNA-DEPENDENT CONTROL OF INTRINSIC EXCITABILITY

In addition to controlling synapse number and efficacy to compensate for local or global activity perturbations, miRNAs can directly regulate membrane excitability, thereby controlling the probability that synaptic inputs trigger action potentials in dendrites and/or axon. For instance, miR-128, which is highly abundant in the mammalian brain, regulates neuronal excitability and motor behavior in the mouse by downregulating the expression of various ion channels and signaling components of the extracellular signal-regulated kinase ERK2 network (Tan et al., 2013). Interestingly, a reduction in miR-128 expression causes increased motor activity and fatal epilepsy in mice. While it would be interesting to see to what extent variations in network activity affect miR-128 expression, these finding suggests that the level of miR-128 is tightly regulated to maintain the neuronal firing rate (Tan et al., 2013).

miRNAs have been involved in the control of intrinsic excitability through the regulation of voltage-gated calcium channel. In the context of chronic pain, miR-103 regulates the expression of the three subunits of the Cav1.2-comprising L-type calcium channel in rat spinal cord neurons, thereby modulating sensitization to pain. Moreover, miR-103 was downregulated in neuropathic rats and miR-103 intrathecal applications successfully relieved pain, thus identifying miR-103 as a possible therapeutic target in neuropathic chronic pain (Favereaux et al., 2011).

Another example is miR-129 which not only controls homeostatic downscaling by targeting Atp2b4 and Dcx (see above; Rajman et al., 2017) but also regulates the dendritic expression of the *Shaker*-like potassium channel Kv1.1 (Sosanya et al., 2013). Kv1.1 is a dendrotoxin-sensitive voltage gated potassium channel that is expressed in the axon but also in dendrites (Raab-Graham et al., 2006; Sosanya et al., 2013). A proposed mechanism involves miR-129 and the mRNA binding protein HuD which binds to Kv1.1 mRNA, depending on mTORC1 kinase activity to repress or enhance Kv1.1 expression, respectively (Sosanya et al., 2013). Interestingly, miR-129-mediated translational repression of Kv1.1 is enhanced 3 weeks after *status epilepticus* in rats, suggesting that miR-129 promotes excitability by targeting Kv1.1 and that this mechanism is tightly regulated to maintain neuronal homeostasis (Sosanya et al., 2015). However, that the same miRNA can promote both synaptic downscaling and dendritic excitability suggests the involvement of complex regulations to orchestrate the homeostatic response in time and space.

There is also evidence that the RNA-binding protein Pumilio 2, a key miR-134 target involved in PTX-induced downscaling (see above), controls the homeostasis of membrane excitability in cultured cortical neurons. Indeed, Pumilio 2 expression at the cellular level is increased upon elevating neuronal activity and thereby suppresses translation of the voltage-gated sodium channel transcript Nav1.6 to decrease intrinsic excitability (Driscoll et al., 2013). However, this is at odds with the fact that the prolonged elevation of neuronal activity reduces Pumilio 2 expression locally in the dendritic compartment in a miR-134-dependent manner to promote downscaling (Fiore et al., 2014). Therefore, it is unclear how elevating activity can simultaneously promote the up- and down-regulation of Pumilio 2 to cause decrease in membrane excitability and miR-134-dependent synaptic downscaling, respectively. This suggests the existence of compartmentalized mechanisms, where Pumilio 2 expression might be differently regulated in the cell body versus dendrites, but this remains to be investigated experimentally.

DO miRNAs REGULATE HOMEOSTATIC PLASTICITY LOCALLY?

What makes miRNAs interesting candidates in the regulation of synaptic plasticity is that they potentially control protein synthesis in remote subcellular compartments such as dendrites and synapses to provide an appropriate and targeted

physiological response. While this idea has not been directly tested in the context of HSP, some of the studies discussed above provide indirect evidence that miRNA-dependent homeostatic plasticity requires local regulations, supporting the concept that synapses do not always adapt uniformly and that homeostatic plasticity can operate within autonomous subcellular compartments, and down to single synapses (Thiagarajan et al., 2005; Sutton et al., 2006; Echegoyen et al., 2007; Aoto et al., 2008; Branco et al., 2008; Kim and Tsien, 2008; Maghsoodi et al., 2008; Beique et al., 2011; Wang et al., 2019).

In support of a role for miRNAs in regulating the function of local compartments like synapses, subcellular fractionation and *in situ* hybridization experiments revealed that several miRNAs are present in dendrites, axons or even synapses and that neuronal activity regulates both their abundance and function (Kye et al., 2007; Lugli et al., 2008; Schratt, 2009; Siegel et al., 2009; Natera-Naranjo et al., 2010). Interestingly, the distribution of miRNAs seems to parallel the distribution of their cognate target mRNAs (Kye et al., 2007); such a spatial proximity may enable the efficient regulation of local protein translation to serve a specific function at the right time and place (Kosik, 2016; Park et al., 2019).

What are the mechanisms by which neuronal activity regulates the local amount of miRNAs? While the activity-dependent expression of several miRNAs including miR-132, miR-134 and miR-124 may be regulated at the transcriptional level by transcription factors such like CREB, Mef2 or EVI1 (Fiore et al., 2009; Nudelman et al., 2010; Remenyi et al., 2010; Hou et al., 2015), there is evidence that neuronal activity directly controls the local processing of pre-miRNAs into mature miRNAs at the level of single dendritic spines. Using a fluorescent pre-miRNA sensor to probe Dicer activity, it was recently shown that the local stimulation of single spines through glutamate uncaging promotes the maturation of miR-181a in a NMDAR-dependent manner, leading to the local repression of CamKIIα synthesis (Sambandan et al., 2017). Furthermore, the local abundance of miR-134, previously implicated in PTX-induced downscaling (Fiore et al., 2014), varies depending on spine maturation and activity, while BDNF local stimulation leads to a decrease in the number of miR-134 copies present at the neck of spines (Park et al., 2019). In addition to the local control of miRNA maturation through Dicer, neuronal activity regulate the turnover of the RISC complex itself, which could possibly impact miR-dependent local protein translation in a non-specific way. In particular, the RISC component MOV10 is degraded upon NMDAR activation, which may result in the release of miRNAs from their mRNA targets and de-repress local protein translation (Chendrimada et al., 2007; Banerjee et al., 2009).

However, one important question remains: how specific activity variations can regulate the local expression and/or function of some specific miRNAs and not others in order to achieve the appropriate physiological response? Potential mechanisms involve specific interactions with cognate mRNA targets which could protect miRNAs from degradation (Pitchiaya et al., 2017), storage in P-bodies whose dendritic location is regulated by neuronal activity (Cougot et al., 2008), or interaction with circular RNAs serving as natural miRNA-sponges (Hansen et al., 2013).

CONCLUSION

There is strong evidence that miRNAs contribute to homeostatic plasticity and associated neurological disorders including epilepsy, neuropsychiatric, and neurodegenerative diseases (Mellios and Sur, 2012; Henshall et al., 2016; Quinlan et al., 2017; Rajman et al., 2017) (**Table 1**). However, despite some recent progress, important questions remain. In particular, the signaling pathways linking physiological synaptic activity variations to miRNA function, trafficking, and turnover remain largely unknown, as most of the current knowledge relies on pharmacological manipulations in culture systems. Some effort will thus be required to investigate the role of identified miRNAs in more physiologically relevant systems; the development of new probes and live-imaging tools to track individual RNAs and investigate translation dynamics (Park et al., 2014; Wang et al., 2016; Wu et al., 2016; Yan et al., 2016) should provide new insights into these mechanisms. Equally important will be to investigate whether they regulate inhibitory synapses which also undergo homeostatic plasticity (Kilman et al., 2002; Peng et al., 2010; Rannals and Kapur, 2011), and whether they contribute to the neuron-glia interactions involved in homeostatic plasticity (Stellwagen and Malenka, 2006; Letellier et al., 2016). Finally, considering that miRNAs also control LTP and depression (Hu and Li, 2017), it will be interesting to investigate whether and how miRNAs enable the integration in time and space of both Hebbian and homeostatic plasticity. A better understanding of the miRNA function in synaptic plasticity and the possible links with pathologies will be very helpful in refining promising therapeutic strategies (Wen, 2016; Angelucci et al., 2019).

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frontiers in Cellular Neuroscience

ORIGINAL RESEARCH
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Mild Inactivation of RE-1 Silencing Transcription Factor (REST) Reduces Susceptibility to Kainic Acid-Induced Seizures

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RE-1 Silencing Transcription factor (REST) controls several steps in neural development by modulating the expression of a wide range of neural genes. Alterations in REST expression have been associated with the onset of epilepsy; however, whether such alterations are deleterious or represent a protective homeostatic response remains elusive. To study the impact of REST modulation on seizure propensity, we developed a tool for its negative modulation *in vivo*. The tool is composed of the paired-amphipathic helix 1 (PAH1) domain, a competitive inhibitor of REST activation by mSin3, fused to the light-oxygen-voltage sensing 2 (LOV2) domain of *Avena sativa* phototropin 1, a molecular switch to alternatively hide or expose the PAH1 inhibitor. We employed the C450A and I539E light-independent AsLOV2 variants to mimic the closed (inactive) and open (active) states of LOV2-PAH1, respectively. Recombinant AAV1/2 viral particles (rAAVs) allowed LOV2-PAH1 expression in HEK293T cells and primary neurons, and efficiently transduced hippocampal neurons *in vivo*. mRNA expression analysis revealed an increased expression of several neuronal genes in the hippocampi of mice expressing the open probe. AAV-transduced mice received a single dose of kainic acid (KA), a treatment known to induce a transient increase of REST levels in the hippocampus. Remarkably, mice expressing the active variant displayed a reduced number of KA-induced seizures, which were less severe compared to mice carrying the inactive probe. These data support the validity of our tool to modulate REST activity *in vivo* and the potential impact of REST modulation on epileptogenesis.

Keywords: RE-1 silencing restriction factor (REST), epilepsy, gene transcription, light-oxygen-voltage (LOV) domain, paired-amphipathic helix 1 (PAH1) domain, kainic acid

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INTRODUCTION

The specification of cell identity during central nervous system development is regulated by positive and negative transcriptional regulators that act simultaneously to shape the cell-specific transcriptome. The RE1-silencing transcription factor (REST), also known as neuron-restrictive silencer factor (NRSF), is a transcriptional repressor that binds a specific 21 bp consensus

sequence named repressor element 1 (RE-1; Chong et al., 1995; Schoenherr and Anderson, 1995). REST is a member of the Kruppel-type zinc finger transcription factor family, whose repressive functions are mediated by two repressor domains: the N-terminal domain interacts with Sin3 (Grimes et al., 2000), while the C-terminus recruits CoREST (Andrés et al., 1999; Ballas et al., 2001). In turn, each co-repressor recruits other associated proteins and chromatin remodeling factors, including histone deacetylases (e.g., HDAC1/2), demethylases (e.g., LSD1), and methyltransferases (e.g., G9a) that mediate the transcriptional repression of target genes (Ballas et al., 2005). Genome-wide sequencing analyses identified several thousands putative RE-1 sites (Mortazavi et al., 2006; Johnson et al., 2007; Jothi et al., 2008), most of which are found in neuron-specific genes (Bruce et al., 2004; Johnson et al., 2007; Otto et al., 2007). Indeed, REST represses the expression of various channels, such as sodium (Chong et al., 1995; Pozzi et al., 2013), calcium (Ariano et al., 2010; van Loo et al., 2012) and potassium channels (Cheong et al., 2005). Moreover, it mediates the transcriptional downregulation of the KCC2 chloride transporter, which is involved in the GABAergic switch from excitatory to inhibitory transmission during neuronal maturation (Yeo et al., 2009). Likewise, REST has been shown to downregulate the expression of Grin2b and GluR2, which code for the NMDA and AMPA receptor subunits, respectively (Calderone et al., 2003; Qiang et al., 2005; Rodenas-Ruano et al., 2012), further supporting its fundamental role in the modulation of genes involved in synaptic activity and plasticity. REST is also involved in the control of neurotransmitter release, whereby it represses several genes involved in neurosecretion, like SNAREs (D'Alessandro et al., 2009), and in synaptic vesicle trafficking, like synapsin 1 (Paonessa et al., 2013).

Because of its pleiotropic functions, alterations of REST expression and/or activity have been described in a wide spectrum of disorders, including Alzheimer's (Lu et al., 2014) and Huntington's disease (Zuccato et al., 2003, 2007) and various types of cancer, where it can act as either tumor suppressor or oncogene, depending on the cellular context (Negrini et al., 2013). In the brain, increased REST levels have been observed after epileptic or ischemic insults (Baldelli and Meldolesi, 2015). In epilepsy, the role of REST is still debated. On the one hand, it seems to have a protecting role as it maintains cell homeostasis by downregulating genes like BDNF (Timmusk et al., 1999; Garriga-Canut et al., 2006); on the other hand, it appears to participate in the induction of the disease, mediating epileptogenesis by inhibiting genes such as HCN1, a hyperpolarization-activated, cyclic nucleotide-gated channel, involved in synaptic transmission and neuronal excitability (McClelland et al., 2011, 2014; Patterson et al., 2017). *In vitro* and *in vivo* studies with kainate, an agonist of glutamatergic receptors, have shown the upregulation of REST levels in hippocampal and cortical neurons (Palm et al., 1998; Hu et al., 2011; McClelland et al., 2014), but whether such increase is protective or deleterious is still not understood. In a rat model of global ischemia, REST is strongly upregulated in post-ischemic CA1 neurons, and linked to neuronal death through the suppression of the AMPA receptor subunit GluR2 (Calderone et al., 2003), modulation of calcium permeability and silencing of the μ-opioid receptor 1 (MOR-1; Formisano et al., 2007). The role of REST in the onset and development of epileptogenesis was addressed by inducing the conditional deletion of REST in mice. The progression of kindling-induced seizures was faster in mice bearing the Calcium/calmodulin-dependent protein kinase II (CaMKII)-Cre driven REST deletion, with a concomitant worsening in mossy fiber sprouting (Hu et al., 2011). In contrast, animals bearing the neuron-specific enolase (NSE)-Cre driven REST deletion were characterized by attenuated susceptibility to pentylenetetrazol (PTZ)-induced seizures (Liu et al., 2012). More recently, the transient block of REST activity *via* a decoy strategy enabled the rescue of the memory impairment induced by febrile seizures (Patterson et al., 2017). These conflicting data could be explained by the different seizure models and/or by the different cell populations where REST was deleted. This suggests that REST may have different functions in the signaling pathways activated by the various convulsants, and/or in the various targeted cell types.

In this work, we have addressed the role of REST in the modulation of kainic acid (KA)-induced seizures. To do so, we have exploited a molecular tool composed of the paired-amphipathic helix 1 (PAH1) domain, a competitive inhibitor of REST activation by mSin3, fused to the light-oxygen-voltage sensing 2 (LOV2) domain of *Avena sativa* phototropin 1, a molecular switchable to alternatively hide or expose the PAH1 inhibitor (Paonessa et al., 2016). Our previous work demonstrated that the LOV-PAH1 probe efficiently controls the expression of REST target genes in primary neuronal cultures, thus modulating network excitability *in vitro* (Paonessa et al., 2016). Here, we performed intra-hippocampal injection of AAVs expressing LOV2-PAH1 and showed that a mild and long-term inhibition of REST activity reduces the susceptibility of mice to develop KA-induced seizures *in vivo*. Overall, our data suggest that REST represents a potential target for therapeutic approaches addressed to pathologies characterized by network hyperexcitability, such as epilepsy.

MATERIALS AND METHODS

Materials

All biochemical reagents and drugs were from Sigma-Aldrich unless otherwise specified. Tissue culture reagents and media were from Gibco-Invitrogen (Thermo-Fisher Scientific, Waltham, MA, USA) or Sigma-Aldrich (Milano, Italy).

Animals

All animals used in this study were mice on the C57BL/6 background (Charles River, Calco, Italy). All experiments were carried out in accordance with the guidelines established by the European Community Council (Directive 2010/63/EU of 22 September 2010) and were approved by the Italian Ministry of Health (Authorization #73-2014-PR on Dec 5, 2014). Hippocampal stereotaxic injections (from Bregma: AP 2.2, LAT 1.5; from brain: Z 1.65) were performed on C57Bl6/J (12–24 weeks) male mice and neurons transduced

with either the "closed state" inactive AsLOV2 (C450A)-PAH1 or the "open state" active AsLOV2 (I539E)-PAH1 variant. Anesthesia was induced by brief exposure to 4% isoflurane and maintained by intraperitoneal (IP) injection of the following anesthetic mixture: ketamine 100 mg/kg, medetomidine 0.65 mg/kg, acepromazine 1.5 mg/kg, atropine 0.05 mg/kg. Mice were placed in a stereotaxic frame and the head adjusted to a flat-skull position. A small craniotomy was performed bilaterally at the injection coordinates indicated above and rAAV1/2 particles carrying AsLOV2 (C450A)-PAH1 or AsLOV2 (I539E)-PAH1 were injected in the hippocampus *via* a glass pipette (0.65 μl–0.75 μl/site at a flow rate of 0.1 μl/min). The injection pipette was left in place for at least 5 min at the end of each injection to allow the complete diffusion of the virus. After injection, mice were returned to their home cage and administered with atipamezole (0.65 mg/kg, IP) to speed up recovery from anesthesia. Mice were allowed to recover for at least 4 weeks before behavioral experiments.

Cloning and AAV Production

To obtain pAAV-CMV_AsLOV-His_Ires GFP, 20 ng of pcDNA3.1_AsLOV2_His (Paonessa et al., 2016) were PCR-amplified using Pfu DNA polymerase (© BiotechRabbit, Hennigsdorf Germany), using primers #1 and #2 (see below). PCR conditions were: 95°C, 5 min; (95°C, 30 s; 60°C, 30 s; 72°C, 1 min) for 27 cycles; 72°C, 5 min and 4°C, ∞. PCR products were digested using Bam HI and Sal I enzymes (NEB, Ipswich, MA, USA), cloned directly in pAAV-IRES-hrGFP Vector (Agilent, Santa Clara, CA, USA), digested with the same enzymes and transformed into TOPTEN cells. Positive colonies were verified by DNA sequencing. To obtain pAAV-CMV_AsLOV-PAH-His_Ires GFP, we proceeded as described above, but starting from pcDNA3.1_AsLOV2_PAH1b_His (Paonessa et al., 2016).

Primer #1 (Fw) 5′CCACCATGGGCGAATTCTTG3′
Primer #2 (Rv) 5′ATCCGTCGACTCACTTCAATGGTGATG GTGATGATGAC3′

AAV1/2 expressing pAAV-CMV_AsLOV-His_Ires GFP and pAAV-CMV_AsLOV-PAH-His_Ires GFP were generated as previously described (McClure et al., 2011). Briefly, human embryonic kidney (HEK)293T cells were co-transfected with the required AAV vector together with the plasmids pRV1, pH21 and pHelper using a Ca^{2+} phosphate method. Forty-eight hours post-transfection, cells were harvested and lysed, and viruses purified over heparin columns (GE HealthCare Life Science, Milano, Italy). Viral vectors were titrated at concentrations ranging from 1×10^{11} to 1×10^{12} transducing units (TU)/ml and used at a multiplicity of infection (MOI) of 10,000. The efficiency of infection, estimated by counting neurons expressing GFP protein with respect to the total number of cells stained with DAPI, ranged between 70% and 90%.

Cell Culture and Transfection/Infection

Immortalized Cells

HEK293T cells were cultured in DMEM (#11965-092) supplemented with 10% (vol/vol) fetal bovine serum (FBS), glutamine (2 mM), and antibiotics, in a humidified 5% CO_2 atmosphere at 37°C. For immunostaining experiments, 180,000 cells were seeded on 24-mm coverslips and the day after were transiently transfected with Lipofectamine 2000 (Life Technologies) following standard transfection procedures.

Primary Neurons

Primary cortical neurons were obtained from E18 embryos derived from crosses of C57BL/6 wild type mice. Mice were mated overnight and separated the following morning. The development of the embryos was timed from the detection of a vaginal plug, which was considered day 0.5. Cortices were dissected in ice-cold phosphate-buffered saline (PBS), incubated with trypsin (0.125%) for 15 min at 37°C, and mechanically dissociated. Neurons were plated in Neurobasal medium containing 10% horse serum, 2 mM glutamine and antibiotics (plating medium). After 3 h, the medium was removed and replaced with Neurobasal containing 2% B27 supplement, 2 mM glutamine and antibiotics (maintenance medium). Neurons were infected at 5 DIV. Experiments were performed 12 days after infection (17 DIV).

Western Blotting

Both tissues and cells were lysed in RIPA buffer (10 mM Tris-HCl pH 7.4, 140 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate) supplemented with proteases and phosphatase inhibitors [complete EDTA-free protease inhibitors, Roche Diagnostics (Monza, Italy); serine/threonine phosphatase inhibitor and tyrosine phosphatase inhibitor, Sigma] and equal amounts of proteins were loaded, as determined by BCA Protein Assay kit (Thermo Scientific). SDS-PAGE and western blotting were performed following standard procedures. After incubation with primary antibodies, membranes were incubated with HRP-conjugated secondary antibodies and ECL Prime Western Blotting System (GE Healthcare) and subsequently imaged using a ChemiDoc imaging system (Biorad, Hercules, CA, USA). Densitometric analysis was performed with Image Lab software (Biorad). The following primary antibodies were used for western blotting: rabbit polyclonal anti-REST 1:1,000 (#07-579, Merck-Millipore, Darmstadt, Germany), rabbit polyclonal anti-calnexin 1:50,000 (#ADI-SPA-860, Enzo Life Sciences, Farmingdale, New York, USA), rabbit polyclonal anti-GFP 1:1,000 (#a11122, Invitrogen); mouse monoclonal anti-His 1:1,000 (#sc-57598, Santa Cruz Biotechnologies, Dallas, TX, USA).

Immunocytochemistry, Immunohistochemistry and Confocal Imaging

Cells were fixed in PBS/4% paraformaldehyde (PFA) for 15 min and washed in PBS. Cells were permeabilized with 0.2% Triton-X 100 in PBS for 10 min at room temperature (RT) then incubated with primary antibodies diluted in PBS 1% BSA overnight at 4°C or 2 h at RT. After washes in PBS, cells were incubated with fluorescent secondary antibodies diluted in PBS 1% BSA. After washes, coverslips were mounted with Mowiol. For brain slices, mouse brains were dissected and

fixed overnight in PBS/4% PFA. They were then cryoprotected in 20% and then 30% sucrose, embedded in OCT, frozen in isopentane (−55°C), and stored at −80°C. Coronal sections (18 μm) were cut with a cryostat and stored at −20°C before immunostaining. Sections were rehydrated in PBS for 5 min and then incubated in 1% Triton X-100 in PBS for 5 min. Slices were blocked for 1 h in PBS containing 5% BSA and incubated with primary antibodies for 24 h at 4°C. After washes in PBS, the slices were incubated with secondary antibodies for 2 h at RT, thoroughly washed and mounted on glass slides with Mowiol. All antibodies were diluted in PBS/5% BSA. Confocal images were obtained using a Leica SP8 confocal scan with a 40×/1.3 oil immersion objective and analyzed with the Leica LAS AF software (Leica Microsystem GmbH, Wetzlar, Germany).

The following primary antibodies were used for immunocytochemistry on fixed cells: rabbit polyclonal anti-GFP 1:500 (#a11122, Invitrogen); mouse monoclonal anti-His 1:200 (#ab18184, Abcam, Cambridge, UK). The following primary antibodies were used for immunohistochemistry on brain slices: rabbit polyclonal anti-REST 1:100 (#07-579, Merck-Millipore), mouse monoclonal anti-glial fibrillary acidic protein 1:1,000 (GFAP, #G3893, Sigma-Aldrich), mouse monoclonal anti-Neuronal Nuclei (NeuN, MAB377 Merck-Millipore). Fluorescently conjugated secondary antibodies were from Molecular Probes (Thermo-Fisher Scientific; Alexa Fluor 488, #A11029; Alexa Fluor 546, #A11030; Alexa Fluor 647, #A21450). Hoechst (#B2261, Sigma-Aldrich) was used to stain nuclei.

RNA Extraction, Nanostring Analysis, and Real-Time PCR

Total RNA was extracted with the RNeasy® Microarray Tissue Kit (Qiagen, Hilden, Germany) from the hippocampi of wild type mice expressing either the "closed" or the "open" probes. The corresponding cDNAs were prepared by reverse transcription of 1 μg of RNA using the SuperScript IV Reverse Transcriptase (ThermoFisher) with an oligo-dT primer according to the manufacturer's instructions. The resulting cDNAs were used as a template for RT-qPCR using a C1000 Touch™ Thermal Cycler (BioRad) on a CFX96™Real-Time System following the manufacturer's protocol. Relative gene expression was determined using the ΔΔCT method. To normalize expression data, primers for 10 commonly used housekeeping genes were used, and the normalization factor was determined using the geNorm software, as described (Vandesompele et al., 2002). This led to the selection of the following internal control genes in our assays: glyceraldehyde 3-phosphate dehydrogenase (Gapdh) and actin. Sequences of the primers used are listed in **Supplementary Table S1**.

For the Nanostring analysis of neuronal genes, fluorescently labeled probes were designed and synthesized by Nanostring Technologies (**Supplementary Table S2**). One hundred nanograms total RNA per sample, prepared as described above, was processed by Synlab Italia S.r.l. (Monza, IT) following standard procedures. Data were analyzed by using the nSolver Analysis Software Version 2.5.

KA Injection and Seizure Scoring

To determine the dose-response of KA (**Figure 2C**), C57BL6/J mice were repeatedly injected with unitary doses of KA (5 mg/kg IP, in 0.9% saline) every 10 min and continuously monitored after each injection. Seizure scoring was conducted as described below. To induce seizures in transduced animals, mice received a single IP injection of vehicle or KA (25 mg/kg). In the KA-treated groups, behavioral seizures were evaluated off-line from video recordings taken during 1 h following the injection. Seizure scoring was conducted based on a modified version of Racine scale (Racine, 1972; McLeod et al., 2013) and the following parameters were considered: 0 = immobility, 1 = erratic twitches, 2 = straight tail, 3 = forepaws shaking, 4 = straight tail together with forepaw shaking (one time), 5 = continuously (>2) show an extended tail shake with forepaw shaking, 6 = display full tonic-clonic seizures. In case of severe attacks (severity score 6) the experiment and video recordings were stopped immediately after the attacks. For immunohistochemical and western blotting evaluation of REST induction, mice were sacrificed at the indicated times after KA administration. Dissection for mRNA extraction and Nanostring/qRT-PCR experiments was performed under a stereomicroscope equipped with a fluorescence lamp to isolate the GFP-expressing hippocampal tissue.

Statistics

Data are presented as means ± SEM throughout. D'Agostino's and Pearson's test were used to check the normality of the experimental data. The two-tailed unpaired Student's *t*-test was used to compare two normally distributed sample groups, while one-way ANOVA followed by Tukey's multiple comparison test was used to compare more than two normally distributed sample groups. For datasets of non-normal distribution, the Mann–Whitney *U*-test was used. The occurrence of a given behavioral trait in the mouse population was evaluated using the Chi-squared test. Alpha levels for all tests were 0.05% (95% confidence intervals). Statistical analysis was performed using GraphPad Prism 6 software (GraphPad Software Inc., San Diego, CA, USA).

RESULTS

Cloning and Expression of AsLOV2-Based REST-Inhibiting and Control Probes

To study the impact of REST modulation on epileptogenesis, we developed a tool for its specific inhibition *in vivo*. The tool is composed of the PAH1 domain, fused to the photosensitive light-oxygen-voltage sensing (LOV) 2 domain of *Avena sativa* phototropin 1 (AsLOV2; Paonessa et al., 2016). PAH1 is the REST-interacting region of the mSin3 protein, which is part of the repressive REST complex (Grimes et al., 2000). We previously demonstrated that PAH1 is a competitive REST inhibitor (Paonessa et al., 2016). For *in vivo* transduction, we cloned the sequence coding for the His-tagged fusion protein into an AAV1/2 vector. In the construct, the expression of the probe is driven by the CMV promoter, while the expression

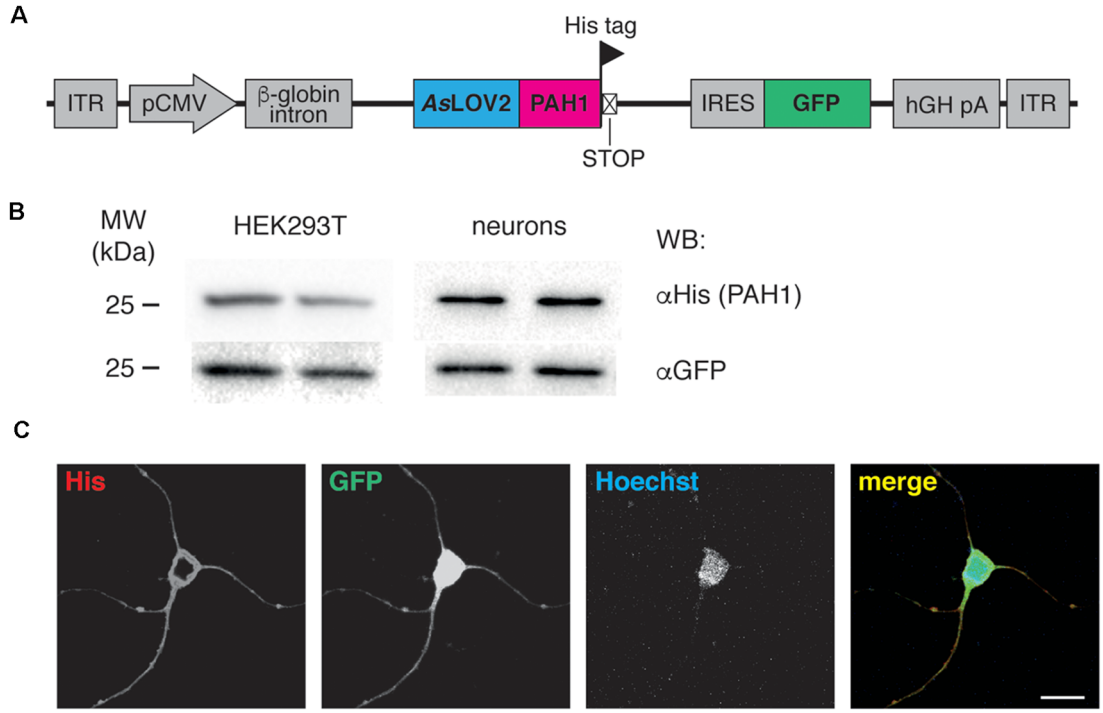

FIGURE 1 | (A) Scheme of the AAV construct used throughout the study. ITR, inverted terminal repeats; pCMV, cytomegalovirus promoter; AsLOV2-PAH1, *Avena sativa* light-oxygen-voltage (LOV) domain 2 fused to the paired-amphipathic helix (PAH) domain 1 of the Sin3A protein; IRES, internal ribosomal entry site; GFP, green fluorescent protein; hGH pA, human growth hormone polyA. The positions of His tag and stop codon are indicated. **(B)** HEK293T cells were transfected with the AsLOV2-PAH1 construct, lysed and processed for western blotting using anti-His and anti-GFP antibodies, as indicated. Cortical neurons were infected at 7 DIV with recombinant AAV1/2 particles expressing AsLOV2-PAH1, lysed at 17 DIV and processed as described above. Duplicate lanes refer to two different cultures/experiments. **(C)** Cortical neurons were infected at 7 DIV with recombinant AAV1/2 particles expressing AsLOV2-PAH1, fixed at 17 DIV and processed for immunocytochemistry using anti-His (red channel), anti-GFP (green channel) and Hoechst to visualize nuclei (blue channel). Scale bar, 20 μm.

of a GFP cassette is controlled by the IRES sequence, allowing the fluorescence detection of transduced neurons (**Figure 1A**). We verified the efficiency of transduction and the expression of the probe by immunoblotting of transfected HEK293T cells and transduced primary neurons (**Figure 1B**). The probe localized in the cytoplasm of neurons, as detected by immunocytochemistry and confocal imaging (**Figure 1C**).

Kainic Acid Injection Induces a Transient Increase in REST Expression

Increased REST levels have been reported in several experimental models of epilepsy (Spencer et al., 2006; Hu et al., 2011). Here, we examined REST protein expression in the hippocampus upon pharmacological induction of epileptogenesis *via* IP injection of (KA, 25 mg/Kg), or saline as a control. We assessed REST protein levels by immunohistochemistry on coronal brain slices followed by confocal microscopy analysis. Slices were co-stained with anti-REST antibodies, anti-NeuN antibodies to detect neurons, anti-GFAP antibodies to detect astrogliosis associated with KA-induced epileptogenesis, and Hoechst to visualize nuclei. We detected increased REST immunoreactivity in slices from KA-treated animals compared to saline-treated control samples (**Figure 2A**, compare right and left panel). The specificity of the anti-REST antibodies was confirmed by the absence of signal in slices incubated with secondary antibodies only, omitting primary antibodies (not shown). The increase in REST expression peaked 24 h after KA injection, as revealed by immunoblotting of lysates obtained from dissected hippocampi (**Figure 2B**). At the dose used, KA induced seizures in all animals, in the majority of cases of severity 5/6 (**Figure 2C**).

Chronic REST Inhibition Leads to Increased Neuronal Gene Expression

To assess the functionality of our construct *in vivo*, AAV1/2-AsLOV2 recombinant viral particles (1 μl/hemisphere) were injected in the hippocampus of adult C57Bl6/J mice (**Figure 3A**) and epifluorescence images obtained from PFA-fixed coronal slices (100 μm) 1 month later. Efficient transduction of hippocampal neurons was detected with a wide (about 1.4 mm) anteroposterior diffusion of the virus from the injection site (**Figure 3B**). Furthermore, animals recovered quickly with no sign of damage at the injection site and no gross behavioral abnormalities, suggesting that the expression of the heterologous protein was well tolerated.

To assess the impact of REST inhibitory modulation by PAH1 on gene transcription, we employed the C450A (Salomon et al., 2000) and I539E (Harper et al., 2004) light-independent AsLOV2 variants to mimic the closed (inactive) and open (active)

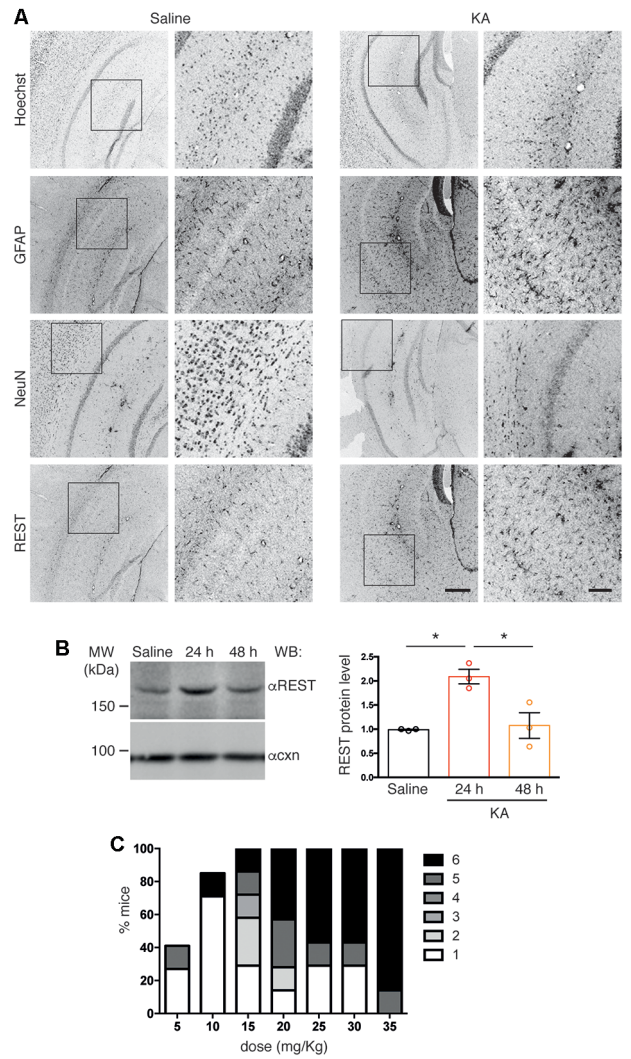

FIGURE 2 | (A) Confocal images of coronal cortico-hippocampal slices derived from mice treated with either saline or kainic acid (KA; 25 mg/kg). Slices were labeled with anti RE-1 Silencing Transcription factor (anti-REST), anti-Neuronal Nuclei (anti-NeuN), and anti-GFAP antibodies, and with Hoechst to visualize nuclei, as indicated. High-magnification images corresponding to the boxed regions are shown for each staining. Scale bars: 300 μm in low-magnification images, 100 μm in high-magnification images. **(B)** The cortico-hippocampal region from saline- (control) or KA-injected mice was dissected at the indicated times after KA injection, lysed and processed for western blotting using anti-REST antibodies (left). Anti-calnexin antibodies were used to verify equal loading. Quantification of immunoreactive bands showed increased REST immunoreactivity at 24 h. One-way ANOVA ($F_{(2,6)} = 12.06$; $p = 0.0079$) followed by Tukey's multiple comparison test ($^*p < 0.05$); $n = 3$. **(C)** Increasing doses of injected KA (5 mg/Kg) in C57BL6/J mice induced seizures of increasing severity in a progressively higher percentage of animals. At 25 mg/Kg all animals reproducibly developed seizures.

state of the AsLOV2 protein, in which the inhibitory peptide is hidden or exposed, respectively. One month after the AAV injection, hippocampi of transduced mice were dissected and a gene expression analysis was performed through the Nanostring nCounter technology (Geiss et al., 2008), which allowed us to quantitatively assess the expression of a panel of neuronal

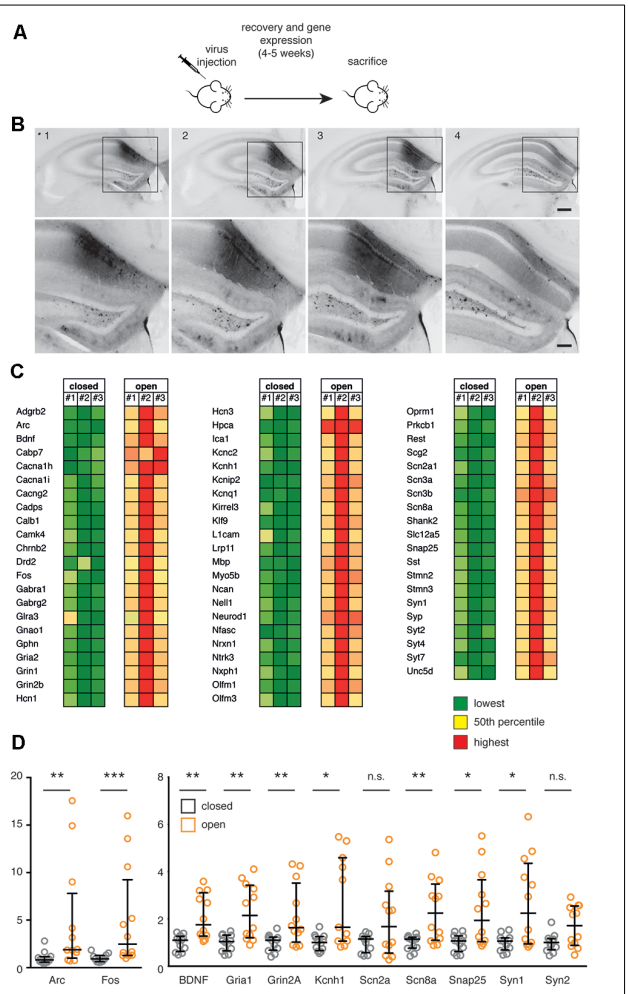

FIGURE 3 | (A) Scheme of the *in vivo* experiment. C57Bl6/J male mice were subjected to stereotaxic injection within the hippocampus with recombinant AAV1/2 particles (0.65 μl-0.75 μl/hemisphere) expressing the constitutively closed [AsLOV2(C450A)-PAH1] or constitutively open [AsLOV2(I539E)-PAH1] probes, let recover for 4–5 weeks and sacrificed for gene expression studies. **(B)** Brain coronal slices (100 μm thickness) were prepared from paraformaldehyde (PFA)-fixed brain of AAV1/2-AsLOV2 injected mice (0.65 μl-0.75 μl/hemisphere), and transduced neurons in the hippocampus were detected by epifluorescence microscopy. Acquired images of four consecutive slices showed a high level of expression at the injection site, and a wide antero/posterior spread of the virus. Image 2 was taken at the injection site (from Bregma: AP 2.2, LAT 1.5; from brain: Z 1.65), the other images were acquired from slices at 100 μm intervals. Scale bars: 200 μm in low-magnification images, 100 μm in high-magnification images. **(C)** mRNA expression from the hippocampus of animals expressing either the constitutively open or the constitutively closed probe was analyzed using the Nanostring nCounter technology. We analyzed $n = 3$ animals per experimental group; a total of 64 REST-target (RE1-containing) genes were analyzed and the values were normalized against five housekeeping genes and for GFP expression. Results are color-coded separately within each gene: green color for low expression, red color for high expression. The complete list of genes and accession numbers is available in **Supplementary Table S2**; the expression values are available in **Supplementary Table S3**. **(D)** The expression of selected genes in the two experimental groups was validated by real time (RT)-PCR. Mann-Whitney test; n.s., non statistically significant ($p > 0.05$), $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; $n = 12$. Bars represent medians; whiskers represent interquartile ranges. The list of genes and accession numbers for RT-PCR is available in **Supplementary Table S1**.

REST-target and non target genes (**Supplementary Table S2**). Interestingly, this analysis revealed a broad upregulation of all genes analyzed in animals expressing the active probe, compared to animals expressing the inactive probe (**Figure 3C**, **Supplementary Table S3**). Of particular interest, we observed the upregulation of synaptic genes (Gphn, L1cam, Nrx1, Shank, Snap25, Syn 1, Syp and several Syt), genes associated with inhibitory (Gabra1, Gabrg2, Glra3, Sst) and excitatory (Gria2, Grin1, Grin2b) transmission, and potassium channels (Hcn1, Hcn3, Kcnc2, Kcnh1, Kcnip2, Kcnq1). The changes in the expression of a subset of these genes and of some related genes were also analyzed through quantitative real-time PCR analysis, confirming the Nanostring results (**Figure 3D**). Altogether, these results suggest that mice expressing the open probe are characterized by an increased expression of a cluster of genes playing key roles in intrinsic excitability and synaptic transmission resulting from an inhibitory influence on the transcriptional repressor activity of REST.

Chronic REST Inhibition Reduces the Susceptibility to KA-Induced Seizures

To address the impact of REST inhibition on epileptogenesis, mice were injected with either the open or the closed probe and after 1 month of recovery, they received a single challenge dose of KA to induce seizures. After KA injection, mice were video-monitored for 1 h to detect seizure onset and severity, and quantified using a modified Racine scale (**Figure 4A**). Remarkably, animals expressing the active probe were less prone to the convulsant action of KA, showing a lower percentage of animals with severe attacks (classified as severity 5 or 6) and an overall reduced seizure score, compared to mice expressing the inactive control probe (**Figures 4B,C**). Interestingly, the analysis of the seizure latency suggests that animals with the active probe experience behavioral signs of the seizure later than mice expressing the inactive probe (**Figure 4D**). In particular, the majority of control animals developed behavioral alterations in the first 20 min, while the majority of mice with the open probe did it in the last 40 min of observation.

DISCUSSION

In this work, we have assessed the impact of long-term, mild inhibition of REST activity on the susceptibility to KA-induced seizures. To do that, rather than directly interfering with the REST-DNA binding, we adopted the strategy of preventing REST activation by mSin3 binding to its N-terminal domain. We previously described that a chimera of the minimal inhibitory peptide PAH1 with the switchable AsLOV domain was able to inhibit REST activity and de-repress the transcription of REST target genes in primary neurons when AsLOV was in the open state (unfolded Jα-helix) and the PAH1 peptide was accordingly exposed (Paonessa et al., 2016). To make this photoswitchable inhibition constitutively active, we generated a point mutant of AsLOV (AsLOV2^{C450A}) in which the Jα-helix is permanently unfolded and used an alternative point mutant (AsLOV2^{I539E}), in which the Jα-helix is permanently folded as a negative control (Paonessa et al., 2016).

As REST expression is increased in KA-induced seizures, we asked whether this increase has a compensatory or rather causal role in the epileptogenic activity triggered by KA. To this aim, the AsLOV2-PAH1 tool, which had been previously shown to work effectively *in vitro* (Paonessa et al., 2016), has been for the first time employed *in vivo*. AsLOV2-PAH1 was efficiently expressed in the hippocampus upon intracranial AAV injection and very well tolerated by the transduced mice, which did not show any gross behavioral alteration upon probe expression. The active probe was correctly working *in vivo* since the hippocampi of transduced mice expressing the active probe were characterized by moderate upregulation of all the REST-target genes, as expected by the chronic effective inhibition of REST repressor activity. Of note, we observed the upregulation of both REST-target and non-target genes, likely reflecting a cascade of effects whereby the initial de-repression of REST-target genes leads to a wider upregulation of neuronal genes. Among up-regulated genes there were clusters playing key roles in intrinsic excitability and synaptic transmission. Genes bi-directionally controlling intrinsic excitability included sodium (Scn2a1, Scn3a, Scn3b, Scn8a, Hcn1, Hcn3), calcium (Cacna1h, Cacna1i, Cacng2) and potassium (Kcnc2, Kcnh1, Kcnip2, Kcnq1) channels. Moreover, a large array of genes playing key roles in both inhibitory and excitatory synaptic transmission were activated, including (i) presynaptic actors of synaptic vesicle trafficking and exocytosis (Syn1, Syp, Syt2, Syt4, Syt7, Scg2, Sst); (ii) glutamate (Gria2, Grin1, Grin2, Shank2) and GABA/glycine (Gabra1, Gabrg2, Gphn, Glra3) receptor complexes; (iii) synaptic adhesion molecules (L1cam, Nrxn1, Nfasc); (iv) immediate early genes (fos, arc); (v) protein kinases involved in transcriptional control and homeostatic plasticity (Camk4; Map2k2, Map3k5, Prkcb1); (vi) neurotrophin signaling (Bdnf, Ntrk3); and (vii) calcium-binding molecules (Calb1, Hpca).

When mice expressing the open and close constructs were challenged with a single KA injection, animals expressing the active probe showed decrease propensity to develop seizures, which were also less severe. In fact, a lower percentage of animals expressing the active probe experienced tonic-clonic attacks, while the vast majority of them showed a mild phenotype, mainly characterized by tail extension and/or forepaws shaking. In addition, control mice displayed behavioral signs of seizure in a much shorter time, again indicating that mice transduced with the active probe were more resistant to the convulsive insult. Since the inhibition of REST activity was constitutive, the administration of the convulsant to trigger seizures found mice in a "low-REST"/"enhanced neuronal phenotype" state, i.e., with upregulation of many gene clusters. Such a global change in the transcriptional profile of neuron-specific genes controlling neuronal communication and network activity, as revealed by our gene expression analysis, is compatible with tighter control of intrinsic excitability and strengthening of both excitatory and inhibitory synaptic connections. This can render neuronal network less susceptible to external stimuli trying to shift the excitation/inhibition balance, by potentiating the push-pull control on depolarizing-hyperpolarizing conductances and on the balance between excitatory and inhibitory synaptic transmission and short-term plasticity. Moreover, the observed

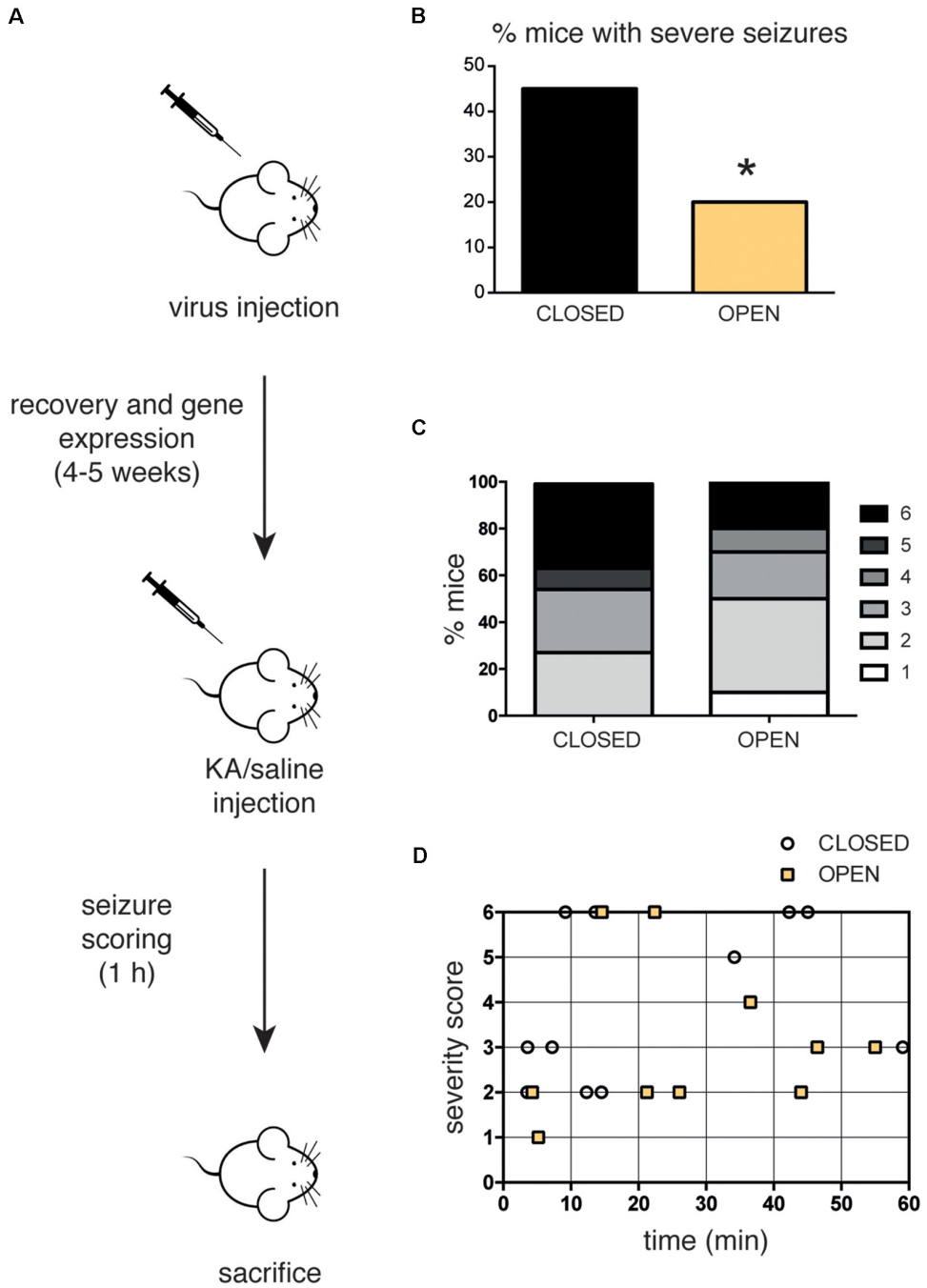

FIGURE 4 | (A) Scheme of the *in vivo* experiment. C57Bl6/J mice (12–24 weeks old) were subjected to stereotaxic injection within the hippocampus of recombinant AAV1/2 particles (0.65 μl-0.75 μl/hemisphere) expressing either the constitutively closed [AsLOV2(C450A)-PAH1] or the constitutively open [AsLOV2(I539E)-PAH1] probes, let recover for 4–5 weeks and subsequently injected with kainate (KA, 25 mg/Kg) to induce seizures. Mice were continuously video-monitored for 1 h after KA injection to track and score seizures. Seizure severity was graded as described in the "Materials and Methods" section. **(B)** When compared to mice injected with the inactive variant, a low percentage of mice injected with the active variant showed severe seizure attacks (score 5/6). **(C)** Bar graphs indicate the percentage of animals for each severity score. Mice expressing the active probe displayed a mild phenotype when compared to control animals. **(D)** Single points represent the individual latency to the highest severity score. The majority of mice expressing the active probe experienced behavioral signs of seizures later than the animals expressing the inactive probe. $n = 11$ mice expressing the closed probe; $n = 10$ mice expressing the open probe. $^*p < 0.05$; Chi-squared test.

upregulation of K^+ channels and of GABA/glycine receptors may represent a brake towards hyperexcitability, making neurons more refractory to the actions of convulsant drugs such as KA. Alterations of REST expression and/or activity have been associated with the onset of epilepsy, although the precise role of this factor in the progression of the pathology is

still debated, and likely depends on the model employed and on the cell type analyzed (Hu et al., 2011; Liu et al., 2012; Patterson et al., 2017). Our findings are consistent with previous studies, which implicated REST in maintaining neuronal homeostasis and reducing the hyperexcitation of the network (Pozzi et al., 2013; Pecoraro-Bisogni et al., 2018; Zullo et al., 2019).

Our results support the notion that REST is actively contributing to the epileptic phenotype, as the chronic inhibition of its activity makes animals less prone to develop seizures upon KA challenge. Future studies will address with more specificity the cell type(s) where REST action is more prominent, by selectively expressing the probe in specific neural cell populations. Moreover, it will be crucial to identify more precisely the time window of REST inhibition that is sufficient to inhibit seizure development, so that appropriate strategies could be designed to exploit REST as molecular target for the treatment of paroxysmal neuropathologies characterized by network hyperexcitability.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the Italian Ministry of Health (Authorization # 73-2014-PR on Dec 5, 2014), and was carried out in accordance with the guidelines established by the European Community Council (Directive 2010/63/EU of 22 September 2010).

AUTHOR CONTRIBUTIONS

EC performed molecular cloning, AAV production, cell and primary neuron infection, biochemistry and confocal imaging, and RT-PCR analysis. FBu performed REST immunohistochemistry and immunoblotting on wild type animals. AR performed immunoblotting, supervised cell and molecular biology and biochemistry experiments. CM performed the *in vivo* experiments and seizure scoring. FC performed Nanostring analysis and wrote the article. FBe supervised the project and wrote the article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fncel.2019.00580/full#supplementary-material.

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frontiers in Cellular Neuroscience

PERSPECTIVE
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Hebbian and Homeostatic Synaptic Plasticity—Do Alterations of One Reflect Enhancement of the Other?

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During the past 50 years, the cellular and molecular mechanisms of synaptic plasticity have been studied in great detail. A plethora of signaling pathways have been identified that account for synaptic changes based on positive and negative feedback mechanisms. Yet, the biological significance of Hebbian synaptic plasticity (= positive feedback) and homeostatic synaptic plasticity (= negative feedback) remains a matter of debate. Specifically, it is unclear how these opposing forms of plasticity, which share common downstream mechanisms, operate in the same networks, neurons, and synapses. Based on the observation that rapid and input-specific homeostatic mechanisms exist, we here discuss a model that is based on signaling pathways that may adjust a balance between Hebbian and homeostatic synaptic plasticity. Hence, "alterations" in Hebbian plasticity may, in fact, resemble "enhanced" homeostasis, which rapidly returns synaptic strength to baseline. In turn, long-lasting experience-dependent synaptic changes may require attenuation of homeostatic mechanisms or the adjustment of homeostatic setpoints at the single-synapse level. In this context, we propose a role for the proteolytic processing of the amyloid precursor protein (APP) in setting a balance between the ability of neurons to express Hebbian and homeostatic synaptic plasticity.

Keywords: hebbian plasticity, homeostatic plasticity, synaptic scaling, amyloid precursor protein, BACE1, APPsα, amyloid-β

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INTRODUCTION

The ability of neural tissue to adapt to specific stimuli through structural, functional and molecular changes plays a fundamental role in complex brain functions such as perception, decision-making, learning and memory (Citri and Malenka, 2008; Bailey et al., 2015). During the past 50 years, considerable effort has been spent to decipher and better understand the cellular and molecular mechanisms of Hebbian synaptic plasticity, which accounts for activity-dependent changes of synaptic weights based on positive feedback mechanisms (Hebb, 1949; Bliss and Lomo, 1973). It is now well-established that Hebbian plasticity resembles fast and lasting input-specific synaptic changes necessary for experience-dependent memory and learning (Bear, 1996; Chen and Tonegawa, 1997; Klintsova and Greenough, 1999). Experimentally, Hebbian mechanisms have been described in detail for excitatory pre- and postsynaptic sites (e.g., Petzoldt et al., 2016; Monday et al., 2018; Scheefhals and MacGillavry, 2018; Buonarati et al., 2019), where, for example, tetanic

electrical stimulation at different frequencies results in the strengthening (long-term potentiation, LTP) or weakening (long-term depression, LTD) of neurotransmission (Bliss and Lomo, 1973; Dudek and Bear, 1992). Meanwhile, evidence has started to emerge for corresponding activity-dependent synaptic changes at GABAergic synapses (Bartos et al., 2011; Rozov et al., 2017; Chiu et al., 2019). Specifically, the plasticity of inhibitory neurotransmission seems to control the ability of neurons to express Hebbian plasticity of excitatory neurotransmission (Letzkus et al., 2015; Zhao et al., 2017).

While feedforward and feedback microcircuits dynamically match afferent excitation to recruited inhibition (Sprekeler, 2017), it has been recognized that, in the absence of physiological constraints, complex systems based solely on positive feedback mechanisms will experience instability—e.g., strong synapses will continue growing, while weakening of synapses will result in synapse elimination (Miller and Mackay, 1994). Indeed, during the past two decades, a plethora of cellular and molecular mechanisms have been identified that maintain neurons in a dynamic functional range by adjusting excitatory and inhibitory synaptic strength in a compensatory manner—i.e., based on negative feedback (Davis and Bezprozvanny, 2001; Marder and Prinz, 2003; Turrigiano and Nelson, 2004; Pozo and Goda, 2010; Keck et al., 2017). Yet, a major unresolved issue in the field concerns the interplay between Hebbian and compensatory—i.e., homeostatic—synaptic plasticity, which share common downstream mechanisms that change and/or adjust excitatory and inhibitory neurotransmission (Turrigiano et al., 1998; Feldman, 2002; Turrigiano and Nelson, 2004; Swanwick et al., 2006; Rannals and Kapur, 2011). Moreover, the biological significance of alterations in Hebbian and/or homeostatic plasticity for pathological brain states remains unclear.

In recent years, these questions have been discussed extensively by leading experts in the field (e.g., Vitureira and Goda, 2013; Fox and Stryker, 2017; Keck et al., 2017; Yee et al., 2017). It has been proposed, for example, that homeostatic plasticity operates on a longer time scale (Turrigiano, 2012; Tononi and Cirelli, 2014; Hengen et al., 2016)—thus not interfering with synaptic changes induced by Hebbian plasticity—and that all synapses of a neuron are adjusted by the same factor in the context of homeostatic "synaptic scaling" to preserve the relative differences between synapses (Turrigiano et al., 1998; Turrigiano, 2008; Vitureira and Goda, 2013). Meanwhile, theoretical modeling work has emphasized the importance of fast homeostatic mechanisms for network stability (Zenke et al., 2013), and robust experimental evidence has been provided for rapid homeostatic plasticity (Keck et al., 2011; Frank, 2014; Li et al., 2014). Furthermore, solid evidence suggests that homeostatic synaptic adaptation can occur locally, in subsets of synapses (e.g., Desai et al., 2002; Kim and Tsien, 2008; Vlachos et al., 2013). These findings indicate that Hebbian and homeostatic synaptic mechanisms may operate in parallel and could thus interfere with each other in the same subset of synapses.

In light of these considerations, it is interesting to note that the effects of classic Hebbian plasticity paradigms—e.g., local tetanic electrical stimulation (Bliss and Lomo, 1973)—have not yet been systematically evaluated for their effects on homeostatic synaptic plasticity induction. Therefore, in this article, we sought to present a "*homeostatic view on classic LTP/LTD experiments*" by highlighting mechanisms which may rapidly affect—and hence set a balance between—Hebbian and homeostatic synaptic plasticity (**Figure 1**). These considerations are put into clinical perspective by discussing the potential role of α- and β-secretase-mediated processing of the amyloid precursor protein (APP) in Hebbian and homeostatic synaptic plasticity (**Figure 2**).

OPPOSING ROLES OF Ca^{2+} SIGNALING IN HEBBIAN AND HOMEOSTATIC SYNAPTIC PLASTICITY

Central mechanisms that regulate the activity-dependent strengthening (or dampening) of excitatory neurotransmission are modification, trafficking and synthesis of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA-Rs) at excitatory postsynapses (Malinow and Malenka, 2002; Diering and Huganir, 2018). Interestingly, both Hebbian and homeostatic synaptic plasticity recruit Ca^{2+}-dependent signaling pathways which lead to characteristic changes in synaptic AMPA-R content and function (Malinow and Malenka, 2002; Song and Huganir, 2002; Derkach et al., 2007; Turrigiano, 2008). However, Ca^{2+} influx *via* N-methyl-D-aspartate receptors (NMDA-Rs) or voltage-gated Ca^{2+} channels (VGCCs) can have opposing effects on postsynaptic AMPA-R content in the context of Hebbian and homeostatic synaptic plasticity (Lee et al., 2000; Diering et al., 2014; Diering and Huganir, 2018).

In the case of LTP induction, for example, tetanic electrical stimulation, which triggers Ca^{2+} influx, can lead to an increase in postsynaptic AMPA-R content and hence potentiation of excitatory neurotransmission (= positive feedback mechanism). Conversely, increased intracellular Ca^{2+} levels are expected to trigger homeostatic synaptic down-scaling, which returns AMPA-R content to baseline (= negative feedback mechanism). Considering such rapid interactions between Hebbian and homeostatic plasticity mechanisms (**Figure 1**), a widely used interpretation of "alterations" in Hebbian plasticity—i.e., failure to persistently change the amplitude or the slope of evoked field excitatory postsynaptic potentials (fEPSPs)—may, in fact, resemble "enhanced" homeostasis, which effectively returns fEPSPs to baseline after the LTP- or LTD-inducing "network perturbation" (see **Figures 1A,C**). Conversely, signaling pathways that block homeostasis or change homeostatic setpoints will result in persisting changes of excitatory neurotransmission (**Figures 1B,C**). We have to concede, however, that molecular signaling pathways that attenuate or adjust local homeostatic plasticity at the level of individual synapses are not well-understood. It is also interesting to speculate in this context that changes in the ability of neurons to express homeostatic plasticity *per se* may suffice to generate

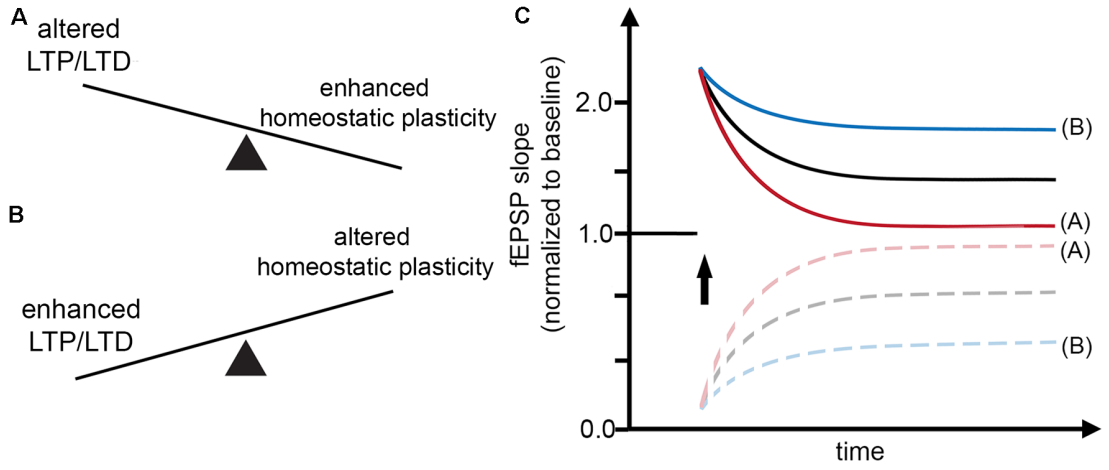

FIGURE 1 | Interaction between Hebbian and homeostatic synaptic plasticity. **(A,B)** Factors may exist which rapidly set a balance between Hebbian and homeostatic synaptic plasticity, thereby affecting the induction and persistence of experience-dependent synaptic changes. **(C)** Alterations in Hebbian plasticity—i.e., long-term potentiation (LTP) or depression (LTD) of evoked field excitatory postsynaptic potentials (fEPSPs; red curve)—may reflect enhanced homeostatic synaptic plasticity. In turn, alterations in homeostatic synaptic plasticity may account for enhanced LTP/LTD (blue curve).

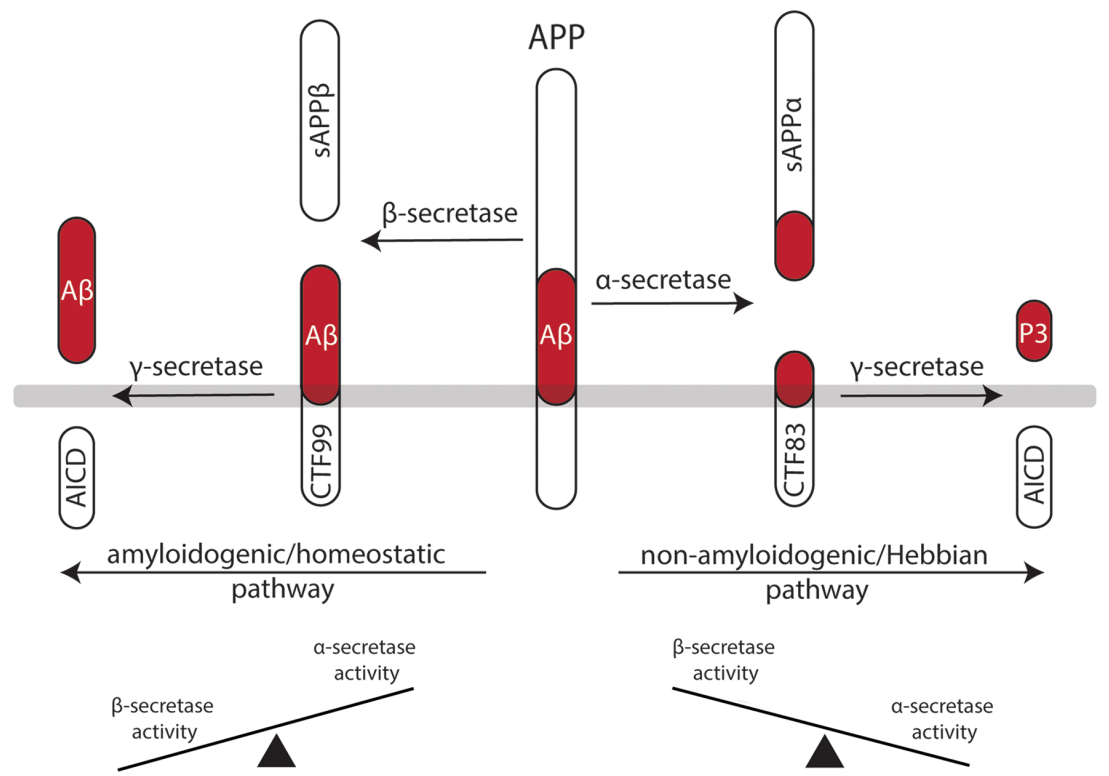

FIGURE 2 | Processing of the amyloid precursor protein (APP) may set a balance between Hebbian and homeostatic synaptic plasticity. Work in recent years has established a firm link between the non-amyloidogenic processing pathway—i.e., APP secreted ectodomain alpha (APPsα)—and the ability of neurons to express LTP of excitatory postsynapses. Likewise, evidence has started to emerge for the role of the amyloidogenic processing pathway—i.e., amyloid-β (Aβ)—in homeostatic synaptic plasticity. Hence, differential processing of APP *via* α- or β-secretases may set a balance between Hebbian and homeostatic synaptic plasticity in neural networks.

Hebbian-like associative plasticity. Indeed, a recent study employed computational modeling to demonstrate associative properties of firing-rate homeostasis in recurrent neuronal networks (Gallinaro and Rotter, 2018).

ROLE OF DOPAMINE IN HOMEOSTATIC SYNAPTIC PLASTICITY

Based on the above considerations, we recently tested for the role of dopamine in homeostatic synaptic plasticity (Strehl et al., 2018). We reasoned that neuromodulators which promote Hebbian plasticity (Otani et al., 2003; Mu et al., 2011; Sheynikhovich et al., 2013; Broussard et al., 2016) may also act by blocking the ability of neurons to express homeostatic synaptic plasticity. Indeed, we were able to demonstrate that dopamine blocks homeostatic plasticity of excitatory neurotransmission in entorhino-hippocampal tissue cultures (Strehl et al., 2018). Pharmacological activation of $D_{1/5}$ receptors, but not $D_{2/3}$ receptors, mimicked the effects of dopamine on homeostatic plasticity. These findings raise the intriguing possibility that dopamine may act as a permissive factor that promotes Hebbian plasticity, at least in part, by blocking homeostasis. Interestingly, the "anti-homeostatic" effects of dopamine were only observed in immature neurons during early postnatal development (Strehl et al., 2018). Hence, specific factors may exist which adjust homeostatic plasticity in specific cells depending on the state of the neural network. It remains to be shown, however, whether dopamine indeed promotes Hebbian plasticity by attenuating homeostatic plasticity at the level of individual synapses and whether dopamine acts on neurons or glia cells (or both) to assert its differential effects on plasticity. Regardless of these considerations, these results call for a re-evaluation of the available LTP/LTD literature and a systematic assessment of well-known "LTP-/LTD-promoting or -blocking factors" in homeostatic synaptic plasticity. As an example that is of considerable clinical relevance, we here discuss the potential role of APP processing in setting a balance between Hebbian and homeostatic synaptic plasticity.

THE ROLE OF THE AMYLOID PRECURSOR PROTEIN IN SYNAPTIC PLASTICITY

Work in recent years has established a firm link between APP and structural and functional plasticity (comprehensively reviewed in Müller et al., 2017). These studies are based on experiments using APP-deficient mice, or mice in which the APP gene has been genetically modified (Dawson et al., 1999; Magara et al., 1999; Seabrook et al., 1999; Turner et al., 2003; Herms et al., 2004). Historically, the majority of studies in the field have focused on addressing the role of APP and its cleavage products in Hebbian plasticity. More recently, some evidence has supported its involvement in homeostatic synaptic plasticity (Jang and Chung, 2016; Styr and Slutsky, 2018).

APP is a type I transmembrane protein ubiquitously expressed in all mammalian tissues (Müller-Hill and Beyreuther, 1989; Müller et al., 2017). It is differentially processed by secretases *via* two pathways (**Figure 2**): the *amyloidogenic processing pathway* generates amyloid-β (Aβ) peptides, which are implicated in the pathogenesis of Alzheimer's disease (AD), while the *non-amyloidogenic processing pathway* produces the neuroprotective soluble ectodomain APPsα (Turner et al., 2003). In the amyloidogenic pathway, APP is cleaved by β-site APP cleaving enzyme (BACE1), which releases APP soluble fragment beta (APPsβ), followed by γ-secretase processing, which generates Aβ fragments and the APP intracellular domain (AICD; Vassar et al., 1999; Van Der Kant and Goldstein, 2015). In contrast, the non-amyloidogenic processing pathway recruits α-secretases releasing APPsα, again followed by γ-secretases that produce the P3 peptide and AICD (O'Brien and Wong, 2011; Van Der Kant and Goldstein, 2015).

ROLE OF THE NON-AMYLOIDOGENIC PATHWAY IN SYNAPTIC PLASTICITY

APP-deficient mice show alterations in dendritic morphologies and dendritic spine counts (Perez et al., 1997; Lee et al., 2010; Tyan et al., 2012; Weyer et al., 2014). These structural defects have been linked to alterations in LTP and deficits in learning and memory (Dawson et al., 1999; Hick et al., 2015). Interestingly, APPsα rescues several of the deficits of $APP^{-/-}$ animals, while APPsβ does not have such a positive effect on Hebbian plasticity (Ring et al., 2007; Hick et al., 2015). Consistent with this suggestion, enhanced LTP is observed in APPsα-treated acute brain slices prepared from rats (Ishida et al., 1997), and behavioral learning is augmented when mice are injected with APPsα (Meziane et al., 1998). Moreover, pharmacologic inhibition of α-secretase activity impairs LTP in rats, which can be rescued by APPsα (Taylor et al., 2008). This line of evidence suggests that APPsα secretion seems to be activity-dependent—that is, LTP-inducing protocols lead to an increase in APPsα (Nitsch et al., 1992; Fazeli et al., 1994). Therefore, it has been proposed that the non-amyloidogenic processing pathway plays an important role in mediating Hebbian synaptic plasticity (**Figure 2**). However, it should be clearly stated that APPsα has not yet been tested in the context of homeostatic synaptic plasticity. It thus remains to be shown whether some of the "positive" effects of APPsα on activity-dependent structural and functional plasticity are also mediated by its ability to modulate—i.e., to attenuate—homeostatic plasticity mechanisms.

ROLE OF THE AMYLOIDOGENIC PATHWAY IN SYNAPTIC PLASTICITY

The role of APP processing *via* the amyloidogenic pathway has been studied in detail for its pathogenic role in neurodegeneration (Goldsworthy and Vallence, 2013; Nieweg et al., 2015; Gupta and Goyal, 2016; Chen et al., 2017; Youn et al., 2019). What remains less understood is the physiological role of the amyloidogenic processing pathway and Aβ.

It seems well-established that elevated concentrations of Aβ are "synaptotoxic" by hindering the ability of neurons to express LTP, thereby having detrimental effects on learning and memory (Chiba et al., 2009; Jo et al., 2011; Samidurai et al., 2018). In this context, it has been shown that Aβ interferes with neural Ca^{2+} signaling—i.e., it blocks NMDA-Rs and Ca^{2+}/calmodulin-dependent protein kinase II (CamKII; Zhao et al., 2004; Townsend et al., 2007; Gu et al., 2009; but see the work in Opazo et al., 2018, which suggests that Aβ

activates CamKII). Similar to APPsα, an increase in synaptic activity and NMDA-R stimulation can also lead to an increase in Aβ production (Kamenetz et al., 2003; Lesné et al., 2005). Thus, it has been proposed that an increase in Aβ may act as a negative feedback mechanism by blocking Hebbian synaptic plasticity. In light of the herein proposed model (**Figure 1**), Aβ may also act by promoting homeostatic synaptic plasticity (see **Figure 1**).

Indeed, evidence has started to emerge for a physiological role of Aβ in homeostatic synaptic plasticity. For example, the AMPA-R scaffolding protein PICK1 mediates homeostatic synaptic plasticity (Anggono et al., 2011) and has been linked to Aβ-mediated "alterations" in synaptic plasticity (Alfonso et al., 2014). Similar evidence exists for interaction between Aβ and PSD-95 (Roselli et al., 2005; Sun and Turrigiano, 2011), GKAP (Roselli et al., 2011; Shin et al., 2012), calcineurin (D'Amelio et al., 2011; Kim and Ziff, 2014) and $STEP_{61}$ (Kurup et al., 2010). Finally, BDNF and TNFα, which have been firmly linked to homeostatic synaptic plasticity (Rutherford et al., 1998; Stellwagen and Malenka, 2006; Becker et al., 2015), seem to be dysregulated in the AD brain (Fillit et al., 1991; Phillips et al., 1991). Along this line of evidence, a role for microglia in Aβ-mediated alterations in complex brain function has been suggested (Kitazawa et al., 2004; Hansen et al., 2018; Kinney et al., 2018; Hemonnot et al., 2019). However, it is important to note that the majority of these findings are based on experiments employing transgenic mouse models of AD or high concentrations of Aβ. Hence, direct experimental evidence for a physiological role of APP/Aβ in homeostatic synaptic plasticity is currently missing (**Figure 2**).

CLINICAL IMPLICATIONS AND PERSPECTIVE

Considering the detrimental effects of Aβ in Hebbian synaptic plasticity together with promising results in experiments employing a mouse model that expressed familial mutant APP in the absence of BACE1 (Cai et al., 2001; Luo et al., 2001; Roberds et al., 2001), pharmacologic inhibition of BACE1 has been tested as a potential treatment for the cognitive decline in AD (Yan and Vassar, 2014; Coimbra et al., 2018). Indeed, BACE1 inhibitors successfully lowered Aβ levels detected in the cerebrospinal fluid of AD patients (Kennedy et al., 2016; Egan et al., 2018). However, major clinical trials were discontinued due to a series of adverse effects or no improvement and even accelerated cognitive decline in patients (Coimbra et al., 2018; Egan et al., 2019). On the same note, mice lacking BACE1 showed increased neural excitability and spontaneous seizure activity (Hitt et al., 2010; Hu et al., 2010; Zhu et al., 2018; Vnencak et al., 2019), which have been linked to impaired homeostatic mechanisms (Wondolowski and Dickman, 2013; González et al., 2015). Although it is clear that BACE1 targets several other substrates in the nervous system (Barão et al., 2016), these observations support the notion that some of the adverse effects of clinically used BACE1 inhibitors could be explained by an impairment of Aβ-mediated homeostatic synaptic plasticity.

Hence, it will be important to evaluate the significance of APP processing *via* the amyloidogenic and non-amyloidogenic processing pathways in homeostatic synaptic plasticity. We are confident that a systematic assessment of "pro-homeostatic" effects of Aβ and possible "anti-homeostatic" effects of APPsα will provide new and important insights into the intricate interplay between Hebbian and homeostatic synaptic plasticity. These findings may also be of relevance for the development of new therapeutic strategies in neurological and psychiatric diseases associated with alterations in APP processing or increased Aβ levels.

AUTHOR CONTRIBUTIONS

CG and AV wrote this manuscript and prepared the figures.

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Emerging Roles of Activity-Dependent Alternative Splicing in Homeostatic Plasticity

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Homeostatic plasticity refers to the ability of neuronal networks to stabilize their activity in the face of external perturbations. Most forms of homeostatic plasticity ultimately depend on changes in the expression or activity of ion channels and synaptic proteins, which may occur at the gene, transcript, or protein level. The most extensively investigated homeostatic mechanisms entail adaptations in protein function or localization following activity-dependent posttranslational modifications. Numerous studies have also highlighted how homeostatic plasticity can be achieved by adjusting local protein translation at synapses or transcription of specific genes in the nucleus. In comparison, little attention has been devoted to whether and how alternative splicing (AS) of pre-mRNAs underlies some forms of homeostatic plasticity. AS not only expands proteome diversity but also contributes to the spatiotemporal dynamics of mRNA transcripts. Prominent in the brain where it can be regulated by neuronal activity, it is a flexible process, tightly controlled by a multitude of factors. Given its extensive use and versatility in optimizing the function of ion channels and synaptic proteins, we argue that AS is ideally suited to achieve homeostatic control of neuronal output. We support this thesis by reviewing emerging evidence linking AS to various forms of homeostatic plasticity: homeostatic intrinsic plasticity, synaptic scaling, and presynaptic homeostatic plasticity. Further, we highlight the relevance of this connection for brain pathologies.

Keywords: alternative splicing, homeostatic plasticity, repressor element 1 silencing transcription factor (REST), homer1, P/Q-type Ca^{2+} channels

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Abbreviations: AMPAR, AMPA-type glutamate receptor; AS, alternative splicing; BDNF, brain-derived neurotrophic factor; CDF, Ca^{2+}-dependent facilitation; CTCF, CCCTC-binding factor; GPCR, G protein-coupled receptor; mEPSCs, miniature excitatory postsynaptic currents; mGluR, metabotropic glutamate receptor; nSR100, Ser/Arg repeat-related protein of 100 kDa; nt, nucleotides; REST, repressor element 1 silencing transcription factor; SNP, single-nucleotide polymorphism; TTX, tetrodotoxin; UTR, untranslated region; VGCC, voltage-gated calcium channel.

INTRODUCTION: FROM GENES TO FUNCTION

Over the last two decades, a vast array of homeostatic plasticity adaptations, which enable neuronal networks to stabilize their activity in the face of external perturbations, have been identified. These involve adjustments in synaptic strength by means of pre- and postsynaptic mechanisms (homeostatic synaptic plasticity) and in intrinsic excitability (homeostatic intrinsic plasticity). Ultimately, both synaptic and intrinsic forms of homeostatic plasticity depend on changes in expression or activity of ion channels and synaptic proteins, which may occur at the gene, transcript, or protein level (**Figure 1**).

By far, the most extensively investigated homeostatic mechanisms involve changes in protein function or localization by means of posttranslational modifications affecting protein–protein interactions and trafficking (reviewed in Turrigiano, 2011; Davis and Müller, 2015; Fernandes and Carvalho, 2016; Cingolani et al., 2019).

Chronic changes in network activity can also be counteracted by regulating protein translation. For example, increased surface expression of the GluA1 subunit of AMPA-type glutamate receptors (AMPARs) compensates blockade of network activity within a few hours. This form of homeostatic synaptic plasticity, known as synaptic upscaling, requires local protein synthesis because it is prevented by dendritic application of the protein synthesis inhibitors anisomycin or emetine (Sutton et al., 2006), and it involves downregulation of miR92a (Letellier et al., 2014; Dubes et al., 2019). Further, the transcription of hundreds of genes was recently shown to be up- or downregulated at early (2 h) and late (24 h) stages of the homeostatic response (Schanzenbächer et al., 2016, 2018).

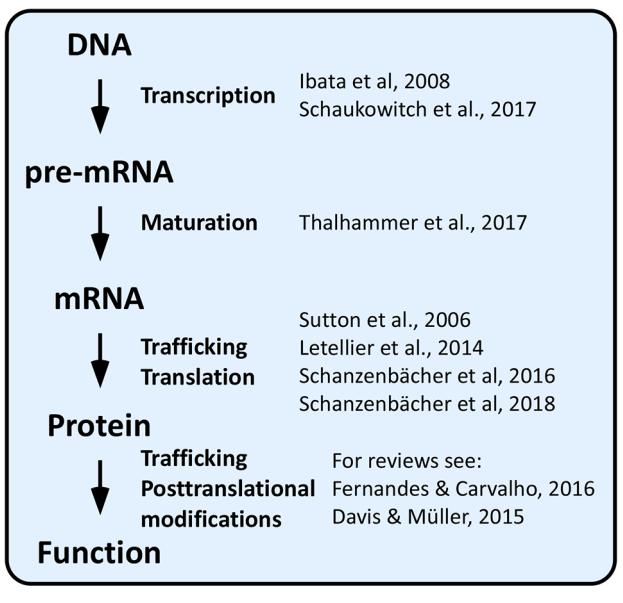

FIGURE 1 | Genes to function in homeostatic plasticity.

Synaptic upscaling following tetrodotoxin (TTX)-induced suppression of network activity is dependent also on gene transcription because the transcription inhibitor actinomycin D (ActD) blocks effectively upscaling of miniature excitatory postsynaptic currents (mEPSCs) and dendritic accumulation of the AMPAR subunit GluA2 (Ibata et al., 2008). More recently, chronic suppression of network activity was shown to alter the transcription of tens of genes, including that for the AMPAR clustering protein neuronal pentraxin-1 (*Nptx1*); Ca^{2+} entry *via* T-type voltage-gated Ca^{2+} channels (VGCCs) appears essential for this signaling pathway (Schaukowitch et al., 2017). Conversely, chronic augmentation of network activity leads to Ca^{2+}-dependent changes in the expression of hundreds of genes (Flavell and Greenberg, 2008; Schaukowitch et al., 2017), some of which, such as brain-derived neurotrophic factor (BDNF), calcineurin, and MeCP2, are known players in homeostatic synaptic plasticity (Fernandes and Carvalho, 2016). Neuronal activity also increases the expression levels of immediate early genes, such as Arc (aka Arg3.1), which induces a counterbalancing internalization of AMPARs (Shepherd et al., 2006) and, when localized in the nucleus, decreases transcription of the AMPAR subunit GluA1, thereby reducing synaptic strength (Korb et al., 2013).

In comparison to the above outlined molecular mechanisms, little attention has thus far been devoted to whether and how homeostatic adaptations are achieved at the level of alternative splicing (AS) of pre-mRNAs (**Figure 1**). As detailed below, this lack of attention may come as a surprise because some AS events are well-known for being controlled by neuronal activity and because AS is ideally suited to optimize protein function to new challenges (Raj and Blencowe, 2015; Vuong et al., 2016; Baralle and Giudice, 2017). Here, we review recent findings linking homeostatic plasticity to AS and discuss the relevance of activity-dependent AS to achieve homeostatic control of neuronal output in health and diseased states.

ALTERNATIVE SPLICING

During RNA maturation, intervening noncoding RNA sequences (introns) are removed while coding sequences (exons) are joined together, thus contributing to transforming a newly transcribed mRNA (pre-mRNA) into a mature mRNA. RNA splicing is performed by a multi-molecular RNA–protein complex, the spliceosome, which binds to specific sequences on the pre-mRNA. These include a donor site (5′ end of the excised intron), an acceptor site (3′ end of the intron), and, upstream of the 3′ site, a polypyrimidine tract and a branch point. For some genes, rather than being univocal, the splicing process creates a range of mature mRNAs, each with a unique exon composition. If translated, these mRNA splice isoforms will produce multiple protein variants with potentially distinct functions. We talk in this case of AS. AS is regulated by *cis*-acting elements (regulatory RNA sequences), which act as splicing enhancers or repressors by recruiting *trans*-acting splicing factors (proteins or ribonucleoproteins) that favor or inhibit different steps of the splicing reaction (Matera and Wang, 2014).

In higher eukaryotes, AS has the potential to convert a limited number of genes into an astounding variety of proteins depending on developmental stage, brain region, and cell types. For example, thousands of mRNA splicing isoforms were found to be different between neurons and glial cells when comparing purified brain cell populations (Zhang et al., 2014). Indeed, some splicing factors display cell-type specific expression (Nguyen et al., 2016; Furlanis and Scheiffele, 2018), while others regulate specific splicing events during brain development (Norris and Calarco, 2012). Transcriptomic and proteomic studies indicate that more than 90% of mammalian genes undergo AS, with the brain exhibiting the most complex repertoire of splice variants (Pan et al., 2008; Wang et al., 2008; Kim et al., 2014; Schreiner et al., 2015). In some cases, as for neurexins and calcium channels, one single gene can give rise to potentially thousands of different mRNA isoforms (Ullrich et al., 1995; Soong et al., 2002; Lipscombe et al., 2013; Schreiner et al., 2014; Treutlein et al., 2014), many of which have been identified at the protein level (Kim et al., 2014; Schreiner et al., 2015). It should also be noted that AS is not limited to diversifying the coding sequence of an mRNA but can also modify the selection of 5' and 3' untranslated regions (UTRs), thus affecting stability, subcellular localization and translation of mRNAs (Hermey et al., 2017; Mauger and Scheiffele, 2017).

In order to be instructive for homeostatic plasticity, AS needs to fulfill two criteria: (i) it must be regulated by neuronal activity; and (ii) the outcome of the splicing process must result in a homeostatic compensation. We will explore the requirement of AS in homeostatic plasticity in the next paragraphs following three exemplary cases.

NSR100, MICROEXONS, AND REST IN HOMEOSTATIC PLASTICITY

Some splicing factors, such as Nova-1/2, Rbfox-1/2/3, Ptbp1/2, and nSR100 are highly enriched in neurons. Among these, the Ser/Arg repeat-related protein of 100 kDa (nSR100, aka SRRM4) binds to intronic enhancer UGC elements close to the 3' splice sites to promote microexon inclusion (**Figure 2A**; Raj and Blencowe, 2015). Microexons are a class of cassette exons (exons that can be included or not in the mature transcript) that tend to be located in surface loops and intrinsically disordered regions. They generally have a length of 9–21 nucleotides (nt), often in multiples of three nt, hence leading to alternative versions of a protein with altered functions, protein–protein interaction motifs, or posttranslational modifications. Microexons are especially important in the brain, where they constitute nearly one third of all neural-regulated splicing events. They are frequently misregulated in the brain of individuals with autism spectrum disorder; this is likely due to increased neuronal activity, often associated with autism spectrum disorder, resulting in a rapid decrease in nSR100 expression and increased skipping of microexons (Irimia et al., 2014; Quesnel-Vallières et al., 2016).

Although generally frame preserving, microexon inclusion promoted by nSR100 can also disrupt the reading frame of a gene. For example, one well-known downstream target of nSR100 is the transcriptional repressor REST (repressor element 1 silencing transcription factor; aka NRSF, neural restrictive silencing factor), which silences a multitude of neural genes (Raj et al., 2011). In this case, nSR100 promotes the inclusion of a 16-nt-long microexon located between the third and fourth exons, leading to a frameshift introducing a stop codon at the beginning of the fourth exon. The resulting isoform, REST4, is truncated and lacks the domains required for transcriptional repression of target genes (Raj et al., 2011). When neuronal activity increases, nSR100 expression is rapidly downregulated (Quesnel-Vallières et al., 2016), resulting in skipping of the 16-nt-long microexon and production of the active isoform of REST (**Figure 2A**). Accordingly, REST is upregulated in primary neuronal cultures after 48–96 h of network hyperactivity, and this decreases the expression of its targets, including the sodium channel $Na_V1.2$, the calcium channel $Ca_V3.2$, and various presynaptic proteins (SNAP-25, Synapsin-1, Synaptotagmin-2, and vGlut-1; van Loo et al., 2012; Pozzi et al., 2013). Downregulation of $Na_V1.2$ makes it more difficult for a neuron to elicit action potentials, thus contributing to homeostatic intrinsic plasticity (Pozzi et al., 2013). Decreased expression of presynaptic proteins correlates with a reduction in the number of docked synaptic vesicles and in the frequency of mEPSCs, thus contributing to presynaptic homeostatic plasticity, a prominent form of homeostatic synaptic plasticity (**Figure 2A**; Pecoraro-Bisogni et al., 2018).

ALTERNATIVE SPLICE ISOFORMS OF HOMER1 IN SYNAPTIC SCALING

The Homer1 gene generates long and short splice isoforms. The major isoforms, Homer1b, Homer1c, and Homer1d, are long, constitutively expressed, and act as scaffold proteins at postsynaptic sites (Fagni et al., 2002; Shiraishi-Yamaguchi and Furuichi, 2007). In response to various stimuli, such as electroconvulsive seizures, cocaine, kainate or nicotine exposure, two truncated isoforms of Homer1, Homer1a and Ania3, which have all the characteristics of immediate early gene products, are rapidly (1–4 h) induced (Brakeman et al., 1997; Kato et al., 1997; Berke et al., 1998; Bottai et al., 2002). This is due to myocyte enhancer factor 2 (MEF2) family transcription factors, which boost transcription of the Homer1 gene and to a concomitant termination of transcription within the large central intron between exons 5 and 6, leading to use of alternative poly(A) sites. Because of this coordinated increase in transcription rate and premature transcription termination, only the short isoforms of Homer1 are induced by neuronal activity (**Figure 2B**; Bottai et al., 2002; Flavell et al., 2008).

The long isoforms of Homer1 consist of two major domains: (i) an N-terminal Enabled/Vasp homology 1 (EVH1) domain, which binds to proline-rich sequences in Group 1 metabotropic glutamate receptors (mGluR1 and 5), inositol-1,4,5-trisphosphate (IP_3) receptors, ryanodine receptors, TRPC1 ion channels, and the scaffold protein Shank; and (ii) a C-terminal coiled-coil (CC) structure followed by leucine zipper motifs, which favor oligomerization of homer proteins (Szumlinski et al., 2006). The long isoforms of Homer1 are

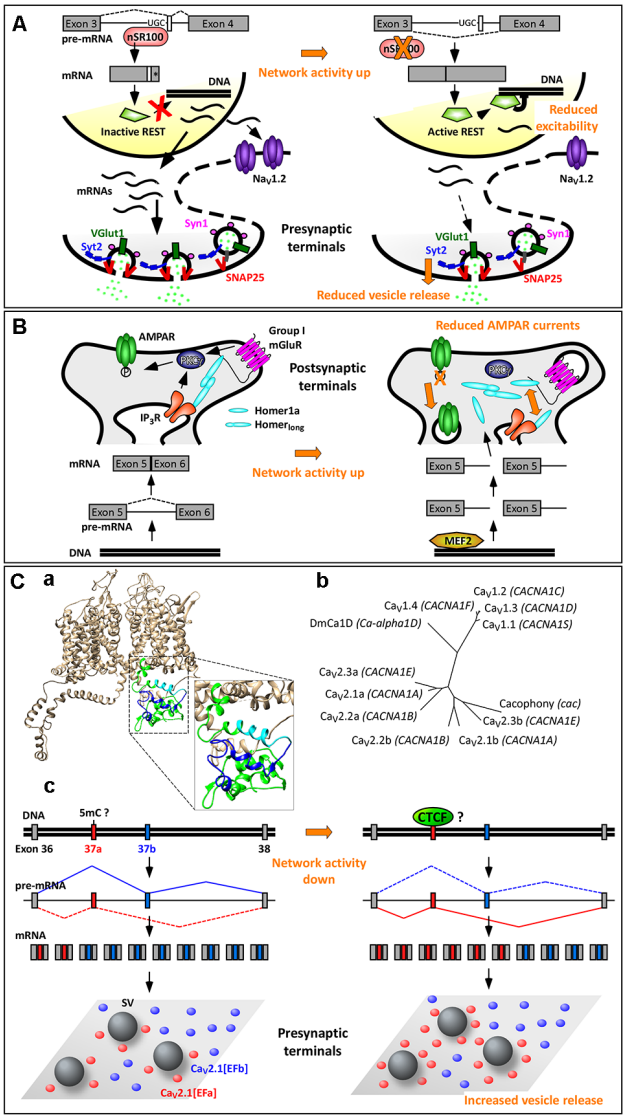

FIGURE 2 | Activity-dependent alternative splicing in homeostatic plasticity. **(A)** A chronic increase in neuronal activity downregulates the expression of the splicing factor nSR100, with consequent skipping of a 16-nt-long microexon in the pre-mRNA of the transcriptional repressor REST (repressor element 1 silencing transcription factor). The resulting REST protein is active and reduces the expression of $Na_V1.2$ and of presynaptic proteins. These two effects contribute to homeostatic intrinsic plasticity and presynaptic homeostatic plasticity, respectively. (*) Indicates a STOP codon. **(B)** The selective induction of the short isoform Homer1a upon increase in neuronal activity is mediated by the transcription factor myocyte enhancer factor 2 (MEF2), which promotes expression of the Homer1 gene, and by a concomitant termination of transcription between exons 5 and 6. Homer1a outcompetes the longer isoforms of Homer1, resulting in dispersion of group 1 mGluRs and dephosphorylation of AMPARs. This contributes to synaptic downscaling. **(C)** Mutually exclusive splicing of P/Q-type Ca^{2+} channels in presynaptic homeostatic plasticity. **(Ca)** Structural model of human $Ca_V2.1$[EFb] (UniProt ID: O00555; Martinez-Ortiz and Cardozo, 2018), highlighting the full C-terminus (green, cyan, blue), the part of the EF-hand-like domain shared between $Ca_V2.1$[EFa] and $Ca_V2.1$[EFb] (E helix; cyan) and the sequence specific to $Ca_V2.1$[EFb] (loop, F helix and downstream residues; blue). **(Cb)** Phylogenetic tree of human Ca_V1 and

(*Continued*)

FIGURE 2 | Continued
Ca_V2 channels and of Cacophony and DmCa1D from *Drosophila melanogaster* for the amino acidic region corresponding to exons 37 of $Ca_V2.1$ (Clustal Omega www.ebi.ac.uk/Tools/msa/clustalo/, rendering using TreeDyb, http://www.phylogeny.fr/one_task.cgi?tasktype=treedyn, Chevenet et al., 2006); UniProt IDs: $Ca_V1.1$: Q13698, aa: 1414–1446; $Ca_V1.2$: Q13936, aa: 1587–1589; $Ca_V1.3$: Q01668, aa: 1497–1529; $Ca_V1.4$: O60840, aa: 1474–1506; $Ca_V2.1b$: O00555, aa: 1843–1875; $Ca_V2.2b$: Q00975, aa: 1741–1773; $Ca_V2.3b$: Q15878, aa: 1756–1788; $Ca_V2.1a$: O00555-4, aa: 1844–1876; Cacophony: P1645, aa: 1370–1402; DmCa1D: Q24270, aa: 1959–1991; sequences for $Ca_V2.2a$ and $Ca_V2.3a$ are as in Thalhammer et al. (2017). The three exons 37a cluster together as do the three exons 37b, suggesting conservation of these mutually exclusive exons across Ca_V2 channels; the corresponding region of Cacophony from *D. melanogaster* is more tightly related to exon 37b. **(Cc)** The increased expression of the isoform $Ca_V2.1$[EFa] upon chronic activity deprivation might occur following demethylation of the exon 37a locus with consequent binding of the chromatin organizer CCCTC-binding factor (CTCF) to it. $Ca_V2.1$[EFa] localizes in close proximity to fuse-competent synaptic vesicles, thereby supporting effectively vesicle release and presynaptic homeostatic plasticity. Drawing of relative exon/intron length is to scale only in (**Cc**); numbers of mRNAs and proteins are not intended to be quantitative.

therefore essential in cross-linking multiple postsynaptic proteins. Conversely, the short isoforms of Homer1 lack the C-terminal domain involved in oligomerization; once induced, they act as dominant-negative regulators disrupting the binding between Homer1 long isoforms and their effectors (Xiao et al., 1998; Kammermeier and Worley, 2007).

Increasing network activity, therefore, upregulates transiently the expression of Homer1a, which, among other things, disrupts the protein–protein interactions clustering group 1 mGluRs at perisynaptic sites. In addition, Homer1a acts as an endogenous allosteric modulator of mGluR1/5; that is, it supports a glutamate-independent activity of these mGluRs (Ango et al., 2001). This is essential in promoting homeostatic downscaling of synaptic AMPARs both *in vitro* and *in vivo* (**Figure 2B**; Hu et al., 2010; Diering et al., 2017), as reviewed elsewhere in this topic (Cingolani et al., 2019).

The expression of Homer1a is increased in the CA1 region of the hippocampus in schizophrenic patients (Matosin et al., 2016) and up- or downregulated in different brain regions of patients with either bipolar disorder or major depression (Leber et al., 2017). Furthermore, in *Fmr1* knockout mice, a model for fragile X syndrome, mGluR5 is preferentially associated to Homer1a, leading to an enhanced glutamate-independent activation of this receptor and consequent neocortical circuit dysfunctions and behavioral abnormalities. Some of these defects are rescued by genetic deletion of Homer1a (Giuffrida et al., 2005; Ronesi et al., 2012), which is consistent with brain function being dependent on an appropriate ratio between short and long isoforms of Homer1.

ALTERNATIVE SPLICING OF P/Q-TYPE Ca^{2+} CHANNELS IN PRESYNAPTIC HOMEOSTATIC PLASTICITY

Mutually exclusive splicing is a form of AS, whereby the splicing of two or more exons is coordinated in such a way that

only one is retained while the others are spliced out from the mature mRNA (**Figure 2Cc**). Mutually exclusive exons are generally highly similar possibly because they originated from exon duplication. However, far from being redundant, they usually allow the formation of protein isoforms that differ in the function of specific domains while preserving the overall structure and size. Indeed, mutually exclusive splicing in many genes is spatially and temporally regulated (Pohl et al., 2013). Recent data indicate that mutually exclusive exons may be much more frequent in mammals than previously thought and that they are overrepresented in genes encoding for ion channels (Hatje et al., 2017). Interestingly, the occurrence of pathogenic single-nucleotide polymorphisms (SNPs) in mutually exclusive and cassette exons is significantly higher than in other types of exons, suggesting that these two forms of AS are especially susceptible to pathogenic mutations. For mutually exclusive splicing, the pathogenic SNPs tend to be present in only one of the two possible exons. Thus, the second mutually exclusive exon cannot normally replace the defective one either because of functional diversification or because of differential spatiotemporal expression patterns (Hatje et al., 2017).

A well-characterized case of mutually exclusive exons occurs in the proximal C-terminus of the pore-forming α_1 subunit of the Ca_V2 VGCCs ($Ca_V2.1$, $Ca_V2.2$, and $Ca_V2.3$; Bourinet et al., 1999; Bell et al., 2004; Gray et al., 2007; Hatje et al., 2017), which serve as primary Ca^{2+} entry for the release of synaptic vesicles at most presynaptic terminals. The 97-nt-long mutually exclusive exons 37a and 37b encode part of an EF-hand-like domain, thus creating two variants of it (EFa and EFb; **Figure 2Ca**; Bourinet et al., 1999; Chaudhuri et al., 2004; Thalhammer et al., 2017). This motif is not specific to Ca_V2 channels but conserved across Ca^{2+} and Na^+ channels (Babitch, 1990; Ben-Johny et al., 2014); in particular, exons 37a and 37b in Ca_V2 channels exhibit a high level of similarity with the corresponding exons in Ca_V1 channels (**Figure 2Cb**).

Which are the functions of the EF-hand-like domain and why do Ca_V2 channels need two variants of it? Three major, not mutually exclusive, functional differences have been proposed. In N-type Ca^{2+} channels ($Ca_V2.2$), mutually exclusive splicing at exons 37 has been shown to regulate sensitivity of the channel to voltage-independent inhibition by G protein-coupled receptors (GPCRs). That is, several GPCRs, including opioid receptors, inhibit $Ca_V2.2$[EFa] but not $Ca_V2.2$[EFb] through kinase phosphorylation of a tyrosine residue (Y1743) present exclusively in the former isoform (Raingo et al., 2007; Andrade et al., 2010). Because $Ca_V2.2$[EFa] is enriched in capsaicin-responsive nociceptors of dorsal root ganglia (Bell et al., 2004), this isoform-specific regulation mediates analgesia, for example, by morphine (Andrade et al., 2010).

In P/Q-type Ca^{2+} channels ($Ca_V2.1$), the two isoforms have been shown to differ in how elevations in intracellular Ca^{2+} regulate the activity of the channel. Specifically, activation of $Ca_V2.1$[EFa], but not $Ca_V2.1$[EFb], is facilitated by preceding Ca^{2+} entry (Ca^{2+}-dependent facilitation, CDF; Chaudhuri et al., 2004). This is in accordance with a large body of evidence indicating that the EF-hand-like domain in the proximal C-terminus of Ca^{2+} and Na^+ channels, rather than binding directly to Ca^{2+}, represents a general transduction element for the regulation of the channel by Ca^{2+}-calmodulin (Peterson et al., 2000; Ben-Johny et al., 2014; Gardill et al., 2018). Calmodulin itself binds, in a Ca^{2+}-independent manner, to downstream domains in the C-terminus of Ca^{2+} and Na^+ channels (Peterson et al., 1999; Zuhlke et al., 1999; Mori et al., 2000; Erickson et al., 2001).

More recently, experiments in native systems have revealed that the two isoforms of $Ca_V2.1$ regulate neurotransmitter release and short-term synaptic plasticity at hippocampal synapses in opposite directions. While $Ca_V2.1$[EFa] promotes synaptic efficacy and short-term synaptic depression, $Ca_V2.1$[EFb] characterizes synapses with low release probability and prominent short-term synaptic facilitation (Thalhammer et al., 2017). This is contrary to what the isoform-specific CDF, as characterized in non-neuronal cells, would have predicted (Chaudhuri et al., 2004; Weyrer et al., 2019); it likely reflects instead a differential spatial relationship of the two isoforms to fuse-competent synaptic vesicles, with a tight and loose coupling configuration for $Ca_V2.1$[EFa] and $Ca_V2.1$[EFb], respectively (**Figure 2Cc**; Thalhammer et al., 2017). More in general, AS of $Ca_V2.1$ might underlie most of the intra- and inter-synaptic differences in nanoscale topographical arrangements of this channel, as recently revealed (Holderith et al., 2012; Nakamura et al., 2015; Rebola et al., 2019).

Whereas the expression of $Ca_V2.1$[EFb] remains relatively constant throughout postnatal development, that of $Ca_V2.1$[EFa] increases postnatally, in parallel with a tightening of the coupling between VGCCs and the neurotransmitter release machinery. As a result, both isoforms are expressed at similar levels in most regions of the adult brain (Bourinet et al., 1999; Soong et al., 2002; Vigues et al., 2002; Chaudhuri et al., 2004; Thalhammer et al., 2017). The developmental upregulation of $Ca_V2.1$[EFa] occurs in rodents between the second and third postnatal week, the same period when ataxic symptoms become apparent in $Ca_V2.1^{-/-}$ knockout mice (Mark et al., 2011). Further, four point mutations associated with episodic ataxia type II have been identified in the exon 37a of *CACNA1A* (the gene for the α_1 subunit of $Ca_V2.1$) in four unrelated families (Graves et al., 2008; Mantuano et al., 2010), while none has been found, to date, in exon 37b, suggesting that $Ca_V2.1$[EFa] might be more relevant for the etiology of episodic ataxia type II than $Ca_V2.1$[EFb].

At the cellular level, while most neurons express both isoforms to various degrees, parvalbumin interneurons, which rely on P/Q-type Ca^{2+} channels to form synapses characterized by nanodomain coupling, high release probability, and short-term synaptic depression (Eggermann et al., 2012), stand out for expressing exclusively $Ca_V2.1$[EFa] (Huntley et al., 2020), again pointing to functional synaptic specialization of the two $Ca_V2.1$ splice isoforms.

Besides these differences in spatiotemporal expression patterns, the relative synaptic abundance of the two isoforms is regulated by network activity in a homeostatic fashion. Specifically, hippocampal neurons increase exclusively the synaptic expression of $Ca_V2.1$[EFa] in response to activity

deprivation. Because this isoform is the more efficient of the two in driving vesicle release, its higher expression levels appear perfectly suited to counteract the decrease in network activity (**Figure 2Cc**; Thalhammer et al., 2017). These findings provide therefore a precise molecular basis for the involvement of P/Q-type Ca^{2+} channels in presynaptic homeostatic plasticity (Frank et al., 2006; Jakawich et al., 2010; Lazarevic et al., 2011; Zhao et al., 2011; Jeans et al., 2017) and highlight the importance of activity-dependent AS in homeostatic synaptic plasticity.

Although it is not known how network activity regulates this splicing event, it has recently been proposed that inclusion of exon 37a or 37b in $Ca_V2.2$ is consequent to differences in chromatin structure and transcription rates, rather than being directly regulated at the mRNA level (Javier et al., 2019; Lopez Soto and Lipscombe, 2020). Because splicing occurs mostly co-transcriptionally (Luco et al., 2011), rapid transcription of *Cacna1b* (the gene for the α_1 subunit of $Ca_V2.2$) would lead to simultaneous availability to the splicing machinery of the two mutually exclusive exons. Direct competition between them would results in inclusion of the downstream stronger exon 37b. Conversely, a slow transcription rate would favor recruitment of the splicing machinery to the first upstream exon 37a, thus leading to inclusion of this weaker exon into the final transcript. Indeed, the zinc finger DNA-binding protein CCCTC-binding factor (CTCF), a well-known organizer of chromatin architecture, binds to the exon 37a locus of *Cacna1b* to promote inclusion of this exon (Javier et al., 2019; Lopez Soto and Lipscombe, 2020). This is likely because CTCF favors the formation of intragenic chromatin loops and slows down the elongation rate of the RNA polymerase II (Pol II; Shukla et al., 2011; Ruiz-Velasco et al., 2017). Importantly, CTCF binding is not constitutive but prevented by methylation of the *Cacna1b* exon 37a locus, which consequently leads to exon 37a exclusion (Javier et al., 2019; Lopez Soto and Lipscombe, 2020).

Because the methylation level of chromosomal DNA is key to both memory formation and homeostatic synaptic plasticity (Day and Sweatt, 2010; Meadows et al., 2015), it is conceivable that activity-dependent methylation and demethylation might regulate also the inclusion of exon 37a in *Cacna1a* during presynaptic homeostatic plasticity. According to databases of chromatin immunoprecipitation followed by sequencing (ChIP-seq[1], ENCODE Project Consortium, 2012), CTCF binds indeed also to the *Cacna1a* exon 37a locus (**Figure 2Cc**).

[1]https://screen.wenglab.org/search/?q=CACNa1A&uuid=0&assembly=GRCh38

DISCUSSION: IMPLICATIONS OF ACTIVITY-DEPENDENT ALTERNATIVE SPLICING FOR BRAIN DISORDERS

Genome-wide transcriptomic studies indicate that AS is more prominent in the brain than in other tissues (Yeo et al., 2004; Pan et al., 2008). Accordingly, defects in AS have been implicated in neurological and neurodegenerative disorders (Raj and Blencowe, 2015; Furlanis and Scheiffele, 2018; Montes et al., 2019). AS defects can originate from mutations that alter either *cis*-acting elements on specific genes or *trans*-acting splicing factors affecting the splicing of multiple transcripts. As discussed briefly in this minireview article, the former mutations are prominent in mutually exclusive and cassette exons involved mostly in monogenic brain pathologies such as episodic ataxia type II, the latter are especially critical for multifactorial brain disorders, for example, for autism spectrum disorder (Gehman et al., 2011; Voineagu et al., 2011; Irimia et al., 2014; Quesnel-Vallières et al., 2016; Gonatopoulos-Pournatzis et al., 2018).

In both cases, to fully understand how defective AS alters circuit and brain function, it is important to consider that some AS events in the brain are regulated by network activity and that the outcome of the splicing process can in turn compensate for changes in activity levels, thus establishing negative feedback loops that make brain function especially resilient to damage. Rather than being direct, the effects of defective AS on brain function are therefore likely to be indirectly mediated by deficient or aberrant homeostatic plasticity mechanisms.

Elucidating the interplay between activity-dependent AS and homeostatic plasticity, as well as implementing new technologies, such as genome editing approaches aimed at correcting pathogenic mutations interfering with AS or at rebalancing splice isoform levels (Gapinske et al., 2018; Konermann et al., 2018; Thalhammer et al., 2018; Yuan et al., 2018), will help us to develop new and improved splicing therapies for brain disorders.

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SYSTEMATIC REVIEW
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The Synaptic Scaling Literature: A Systematic Review of Methodologies and Quality of Reporting

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The maintenance of the excitability of neurons and circuits is a fundamental process for healthy brain functions. One of the main homeostatic mechanisms responsible for such regulation is synaptic scaling. While this type of plasticity is well-characterized through a robust body of literature, there are no systematic evaluations of the methodological and reporting features from these studies. Our review yielded 168 articles directly investigating synaptic scaling mechanisms, which display relatively high impact, with a median impact factor of 7.76 for the publishing journals. Our methodological analysis identified that 86% of the articles made use of inhibitory interventions to induce synaptic scaling, while only 41% of those studies contain excitatory manipulations. To verify the effects of synaptic scaling, the most assessed outcome was miniature excitatory postsynaptic current (mEPSC) recordings, performed in 71% of the articles. We could also observe that the field is mostly focused on mechanistic studies of the synaptic scaling pathways (70%), rather than the interaction with other types of plasticity, such as Hebbian processes (4%). We found that more than half of the articles failed to describe simple features, such as regulatory compliance statements, ethics committee approval, or statements of conflict of interests. In light of these results, we discuss the strengths and pitfalls existing in synaptic scaling literature.

Keywords: synaptic scaling, homeostatic plasticity, systematic review, molecular methods, electrophysiology, risk of bias assessment, quality of reporting

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INTRODUCTION

Animal models are valuable tools for understanding human diseases and physiological mechanisms. However, their application is limited, as just a fraction of the efficacious interventions seems to be translatable to humans (O'Collins et al., 2006). Thus, structured methods of literature synthesis are required to make an objective sense of the large volume of preclinical research and locate the most promising findings. Systematic reviews and meta-analyses are useful tools that can address some of these challenges by providing an objective summary of scientific articles, appraising available evidence, and evaluating the likelihood that a given conclusion is biased (Macleod et al., 2015). For such reasons, the number of systematic studies from preclinical data has been rising in recent years (Vesterinen et al., 2014), mostly focusing on the application of animal models (Sena et al., 2014).

Synaptic scaling is a type of homeostatic plasticity that was first described around 20 years ago (Turrigiano et al., 1998), believed as necessary for proper development and function of neuronal networks (Turrigiano and Nelson, 2004). It is a negative feedback response mechanism to chronic changes in the level of network activity, in which the synaptic strengths of a neuron are modified by regulating synaptic receptors following a universal multiplicative scaling factor. This adjustment happens in a way that the total synaptic input matches the neuron's homeostatic range while preserving the relative differences between synaptic weights (Abbott and Nelson, 2000; Turrigiano, 2012). By a bidirectional interaction with other types of plasticity, it is able to maintain many aspects of neural function and to regulate future synaptic modifications (Fernandes and Carvalho, 2016; Keck et al., 2017a; Moulin et al., 2019).

Moreover, homeostatic plasticity has been shown to influence the pathophysiology of several neuropsychiatric and neurologic disorders, such as intellectual disability (Soden and Chen, 2010), Rett syndrome (Qiu et al., 2012), schizophrenia (Dickman and Davis, 2009), and Alzheimer's disease (Yamamoto et al., 2015). However, despite its relevance, to the best of our knowledge there is no systematic approach to answer questions such as what is impact and reliability of the field, which are the most commonly used techniques, and how the methods are changing over time.

In this study, we performed a systematic review of articles on synaptic scaling to address these issues. Our first goal was to describe important features of the field, such as impact factor distribution and countries where these studies are produced. We investigated which are the popular models for synaptic scaling experiments, followed by an evaluation of the main intervention types to induce homeostatic changes and which outcomes are assessed. We then analyze the reporting of measures to reduce the risk of bias in these studies. We conclude with a discussion on the implications of this research, as well as gaps in the empirical results that limit our understanding of this homeostatic mechanism.

METHODS

Search Strategy

We performed two separate searches in PubMed to find publications related to synaptic scaling and homeostatic plasticity. Our first search used the most established keywords for describing this process ("homeostatic plasticity" OR "synaptic scaling"), which returned 664 articles. We then performed a second search for articles that might have been missed by those specific keywords, combining the most common descriptions of outcomes and methods ("(mEPSC* OR mIPSC* OR patch clamp*) AND (scaling OR homeostat* OR chronic* inhibit* OR chronic* excitat*) NOT review"), which returned 618 studies. Duplicated articles (61) were removed. There were no time constraints on the searches, which were both performed on May 31st, 2018.

Study Selection

The first screening step considered only titles and abstracts, excluding (i) articles not written in English, (ii) articles not presenting original results, such as reviews, and (iii) articles not describing animal experiments using chronic stimulation or inhibition of neurons to study homeostatic synaptic scaling plasticity. This first step was performed by both authors using the Abstrackr online platform (Wallace et al., 2012), and at least one had to include the reference for it to be taken to the next screening stage. If the title and abstract were not clear about the three criteria described above, articles were still included for further screening.

The second screening stage considered the full text of the articles. They were included if they meet the following criteria: (i) described the effects of chronic neuronal stimulation or inhibition on an outcome, (ii) controlled for intensity and time, (iii) used interventions with known effects on synaptic transmission and/or firing of the studied neuronal population, and (iv) investigated changes in neuronal excitability through synaptic homeostatic plasticity, as defined by the objectives and discussion of the article. Despite the subjectivity that is inherent to interpreting phenomena as being due to scaling, our goal was to have a representative sample of the synaptic scaling literature, rather than performing an extensive pursuit of other findings that might correspond to synaptic scaling. After evaluation on these criteria, we used the included articles to extract the type of experiments performed, the study and journal citation metrics, and the reporting of measures to control the risk of bias. At this stage, data for each article were extracted by one of the authors.

Data Extraction

We built Microsoft Excel spreadsheets as a database to include all articles selected in the first screening stage. For those that met inclusion criteria, data obtained from the second screening stage were also stored in this database. The following items were extracted and recorded for the systematic review:

Publication features: PMID; first author's name; journal name; year of publication; country of origin (defined by the corresponding author affiliation); and impact factor of the journal (obtained from the Scimago Journal Rank for the publication year).

Risk of bias assessment: Blinded assessment of outcome; unbiased methods for data selection (the description of any method aiming to diminish the possible bias occurring in data selection, e.g., randomly selecting 10 out of 100 mEPSC recordings to analysis); the presence of sample size or power calculation within the article; statement regarding compliance with regulatory requirements for animal research; statement of local ethics committee approval; statement regarding conflict of interest on the part of the authors. These items were considered present if they were described at any point in the article.

Experimental features: "Direct / Indirect" intervention—whether the article performed a manipulation directly on the neuronal population later assessed for scaling (e.g., assessment of neurons chronically treated with TTX), or on a circuit projecting to the neuronal population tested for scaling, (e.g., monocular deprivation with visual cortex assessment, or entorhinal denervation with hippocampal DG recordings). "Intervention method"—description of the substance(s) or method(s) of intervention used to induce scaling (e.g., TTX,

ChR2, visual deprivation). "Species"—the animal species used in the experiments. "*In vitro*/*in vivo*" and "Model application"–brief descriptions of the model used in the experiments to induce synaptic scaling (e.g., *in vitro* hippocampal primary culture). "Inhibitory / Excitatory" interventions—the presence of inhibitory or excitatory manipulations to induce synaptic scaling. "mEPSC"—the presence of miniature excitatory post-synaptic current recordings. "mIPSC"—the presence of miniature inhibitory post-synaptic current recordings. "Dendritic spines"—the presence of an assessment of dendritic spine density or area. "Synaptic membrane channels"—the presence of an assessment of the transcription or expression of synaptic membrane channels/receptors or their subunits. "Other synaptic proteins"—the presence of an assessment of other synaptic proteins (e.g., PSD95, GAD65, VIAAT). "Effect on Hebbian plasticity"—the presence of an assessment of Hebbian plasticity (e.g., induction of LTP or LTD) after synaptic scaling protocols. "Interference with scaling mechanism"—the presence of experiments studying the effects of interfering with specific mechanisms on scaling (e.g., using pharmacological or genetic interventions to identify the pathways involved in the synaptic scaling). "Firing rate homeostasis"—the presence of neuronal spiking assessment to evaluate the homeostatic effects of the manipulation in the neuronal function. "Multiplicative scaling"—whether the article discusses multiplicative scaling changes in the mPSC amplitudes, and if it is demonstrated by linear fit/ regression of the ranked mPSC amplitude distributions, or by performing the Kolmogorov-Smirnov test after multiplying the cumulative amplitude distribution by a scaling factor.

RESULTS

Article Selection and Inclusion

Articles were screened by combining two search strategies to broaden the detection of relevant studies (see Methods). After the exclusion of duplicates, 1,221 articles were obtained (**Figure 1**). In the first screening step, two investigators examined all articles based on titles and abstracts, and the agreement for exclusions

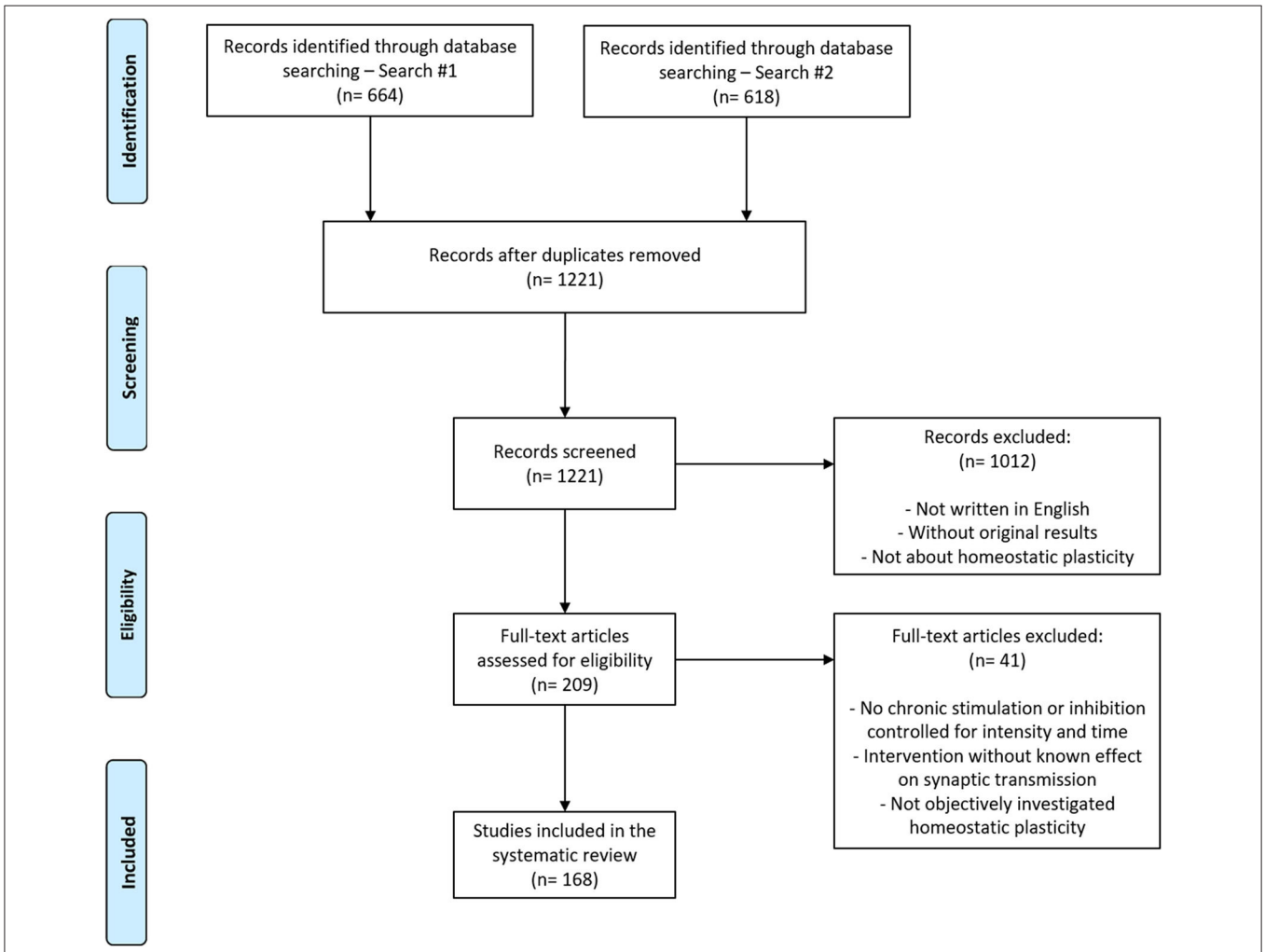

FIGURE 1 | Flowchart of article search and selection. Of the 1,221 articles retrieved from the combination of two search strategies, 168 were included in our analysis after the two-stage screening process (see section Methods for details).

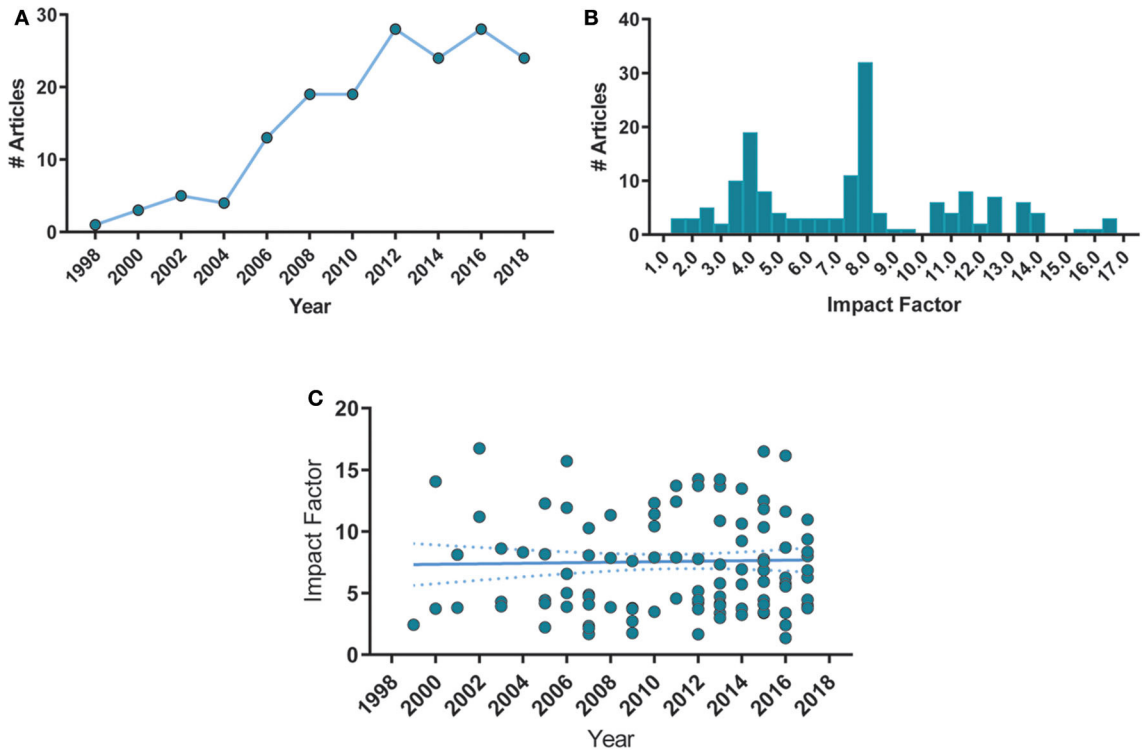

FIGURE 2 | Histogram distributions of articles per publication year and impact factor. **(A)** Number of article publications over time. Each point corresponds to a 2-year bin. Spearman's correlation, $\rho = 0.93$, $p < 0.0001$. **(B)** Number of articles distributed by their respective journals' impact factor, with a bin size of 0.5. Median = 7.76, min = 1.36, max = 16.74, $n = 157$. **(C)** Mean impact factor remained stable over time. Spearman's $\rho = -0.001$, $p = 0.904$. Solid lines represent the linear fit of the data. Dashed lines are the 95% C.I. of the linear fit.

measured on a double-screened sample of 200 articles was 95%. It led to 209 articles selected for full-text screening. Ultimately, 168 articles met all criteria and were considered for further analysis.

Literature Characteristics

First, we analyzed the year of publication of all articles and the distribution of impact factors of their respective journals (**Figure 2**). Impact factors (number of citations divided by the number of citable documents for the previous 2 years) were obtained through the Scimago Journal Rank database corresponding to the year of publication and were unavailable for 11 of the included articles. There was a significant increase in publications over the years (**Figure 2A**), with a median impact factor of 7.76 (**Figure 2B**). Additionally, we noticed that the mean impact factor over the years remained stable (**Figure 2C**). These results suggest that the interest of high-impact journals on the subject has remained elevated over the years. Regarding demographics, more than 80% of studies from our sample were originated from the United States, Germany, and the United Kingdom (**Supplementary Table 1**).

Features of the Experimental Models

Next, we investigated which animal models were mostly employed for synaptic scaling studies, either by the use of the whole organism during *in vivo* trials or as the tissue source for *in vitro* experiments. Rodents were the most prevalent species, as rats were used in 52% of the studies, followed by nearly 40% of the reports employing mice. Interestingly, there is a significant decrease in rat-base testing over time, while the usage of mice significantly grew over the years.

Moreover, *in vitro* models seem to be the approach of choice for the field (83%), largely due to experiments using dissociated-cell cultures, present in almost 60% of our sample, followed by organotypic cultures (18%). For the articles with *in vivo* investigations (18%), most were performed by sensorial manipulations (13%), while direct circuit interventions (e.g., pharmacological or optogenetic stimulation in a given brain area) were present in only 5% of our sample.

Methodological Aspects of Synaptic Scaling Assessment

We analyzed the main experimental features from the sample articles regarding protocols to induce and evaluate synaptic scaling (**Table 2**). We first categorized different kinds of scaling-inducing interventions as excitatory (e.g., bicuculline, picrotoxin) or inhibitory (e.g., TTX, visual deprivation). We also classified the interventions as direct (i.e., applied directly to the neuronal population assessed for homeostatic changes) or indirect (i.e., applied to a pre-synaptic circuitry from the studied neurons). The vast majority of the articles (86%) employ inhibitory

TABLE 1 | Experimental models.

Species	# Articles (%) [95% C.I]	Trend over time (ρ)	*p*-value
Mouse	66 (39.3) [32.2, 46.8]	0.225	0.0034[#]
Rat	87 (51.8) [44.3, 59.2]	−0.283	0.0002[#]
Drosophila	7 (4.2) [2.0, 8.3]	0.172	0.026
Chicken	4 (2.4) [0.9, 5.9]	0.048	0.533
Others	5 (3.0) [1.3, 6.8]	−0.019	0.808
Not described	2 (1.2) [0.3, 4.2]	−0.086	0.263
Model application			
In vitro	140 (83.3) [76.9, 88.2]	0.011	0.888
Dissociated-cells culture	99 (58.9) [51.3, 66.1]	0.009	0.909
Organotypic culture	31 (18.4) [13.3, 25.0]	−0.116	0.135
Acute brain slice	4 (2.4) [0.9, 5.9]	−0.035	0.651
Others	8 (4.7) [2.4, 9.1]	0.122	0.114
In vivo	31 (18.4) [13.3, 25.0]	0.021	0.791
Sensorial manipulations	22 (13.1) [8.8, 19.3]	0.053	0.496
Brain circuitry intervention	9 (5.4) [2.6, 10.2]	−0.044	0.573

The columns show the number of articles reporting each item, with percentages relative to the total number of articles included (n = 168 for all items), and their 95% confidence intervals. Spearman's correlation was used to estimate the ρ coefficient and p-values for model use over time. [#]Significantly correlated with time (α = 0.0085 for species correlations and α = 0.0064 for model application correlations, Bonferroni correction for multiple comparisons).

TABLE 2 | Intervention and assessment features.

Intervention to induce scaling	# Articles (%) [95% C.I]	Trend over time (ρ)	*p*-value
Inhibition	145 (86.3) [81.1, 91.5]	−0.061	0.435
Excitation	69 (41.1) [33.7, 48.5]	0.046	0.558
Direct	149 (88.7) [83.9, 93.5]	−0.070	0.370
Indirect	21 (12.5) [7.5, 17.5]	0.073	0.344
Outcome evaluated			
mEPSCs	120 (71.4) [64.6, 78.2]	0.115	0.137
mIPSCs	25 (14.9) [9.5, 20.2]	−0.089	0.253
Dendritic spines	13 (7.7) [3.7, 11.8]	0.024	0.756
Synaptic channels	67 (39.9) [32.5, 47.3]	0.036	0.646
Other synaptic proteins	27 (16.1) [10.5, 21.6]	−0.128	0.098
Additional features			
Interference with scaling mechanism	118 (70.2) [63.3, 77.1]	0.314	<0.0001[#]
Effect on Hebbian plasticity	7 (4.2) [1.2, 7.2]	0.066	0.394
Firing rate homeostasis	40 (23.8) [1.8, 30.8]	−0.097	0.212
Multiplicative scaling	49 (29.2) [22.5, 36.8]	−0.018	0.815

The number of articles that contains a reported item, with the percentages relative to the total quantity of articles included (n = 168), and the 95% confidence interval is shown. Spearman correlation test was used to estimate the ρ coefficient and p-values for the application of the methods over time. [#]Significantly correlated with time (α = 0.0046 after Bonferroni correction for multiple comparisons).

interventions to induce synaptic scaling, while less than half of the studies (41%) contain excitatory ones. The most popular inhibitory manipulation was TTX, used in 55% of the articles, while bicuculline was the most used intervention for neuronal excitation (26%). A list of the main manipulations used in the studies can be found on **Supplementary Table 2**. We observed that these interventions are mostly administered directly to the same neurons from which the scaling outcomes are measured (89%), rather than indirectly via other circuits or sensorial systems (12.5%).

Next, we assessed the widespread outcomes tested after inducing synaptic scaling, such as miniature excitatory and inhibitory postsynaptic currents (mEPSCs/ mIPSCs), present in 71% and 15% of the reports, respectively; analyses of dendritic spines (density, area, or volume) (8%); and relative changes in synaptic channels (40%) or other synaptic proteins (16%).

To investigate the number of reports that consider the specific components of synaptic scaling, we registered whether the articles had protocols for interfering with mechanisms or pathways of scaling processes (e.g., inhibition of a given transcription factor to study its effects) (70%); if they studied the influence of homeostatic plasticity on Hebbian-like mechanisms (e.g., by inducing LTP or LTD after scaling protocols) (4%); and the assessment of hallmark characteristics, such as whether firing rate homeostasis is observed (24%) or if the changes in mPSC follow multiplicative changes (29%).

The description of methods for analyzing multiplicative scaling was also evaluated (**Supplementary Figure 1**). Within the articles with this feature, performing a Kolmogorov-Smirnov test after multiplying the amplitude distribution by a scaling factor was present in 29% of the reports, while linear regression/ correlation analysis for the ranked amplitudes was described in 26% on them. The combined use of these analyses was observed in 33% of the articles.

Associations Between Experimental Procedures

We then calculated the correlations between the different methodological aspects of synaptic scaling experiments (**Figure 3A**). We observed that the reporting of inhibitory interventions to induce scaling has a negative correlation with the reporting of excitatory manipulations ($\rho = -0.48$, $p < 0.0001$), indicating that most studies are usually limited to one of the approaches. Assessment of dendritic spines tends to be less present when inhibitory interventions are used ($\rho = -0.27$, $p = 0.0003$), while analyses of synaptic channels or receptors are more common in studies with excitatory interventions ($\rho = 0.23$, $p = 0.002$). Studies measuring synaptic channels are also more likely to analyze other synaptic proteins ($\rho = 0.27$, $p = 0.0004$). Also, articles using manipulations interfering with synaptic scaling mechanisms are more likely to report mEPSCs measurements ($\rho = 0.28$, $p = 0.0002$), and quantifications of synaptic membrane channels ($\rho = 0.29$, $p = 0.0001$).

We then investigated the relationship between the choice of experimental models and methodology. No significant correlation was found between the use of either mice or rats, the most popular species, and assessment features. However, when analyzing specific experimental models, many methodological preferences were identified. First, dissociated-cell cultures were

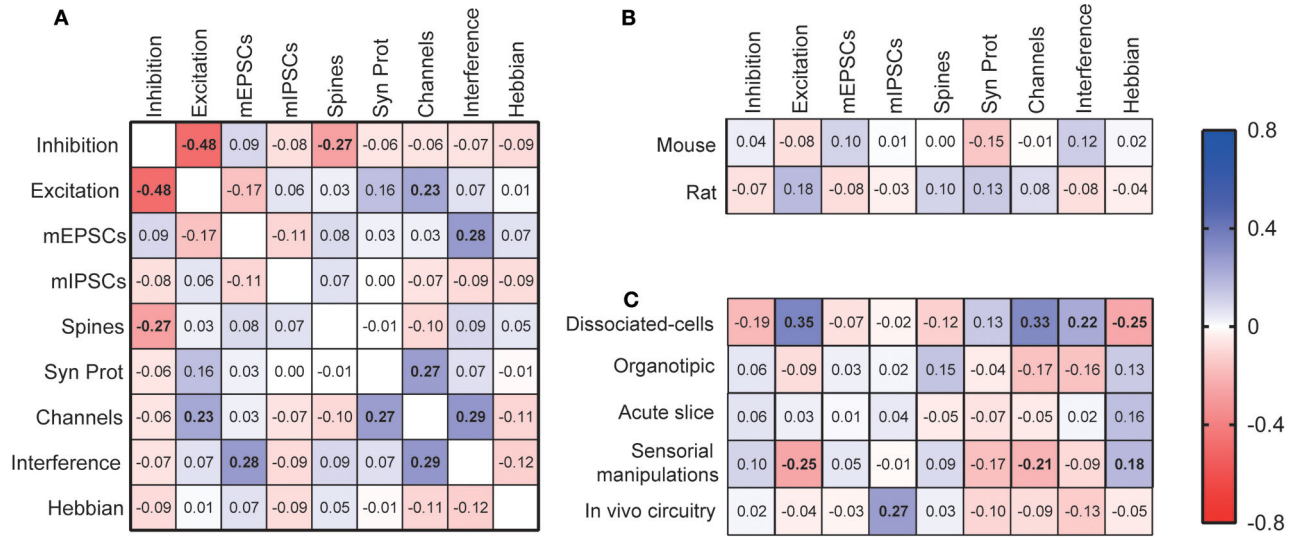

FIGURE 3 | Correlations among experimental features. **(A)** Correlation matrix for the use of different synaptic scaling assessment methods. These features were also correlated with the most used species **(B)** and experimental models **(C)**. "Inhibition"—inhibitory scaling interventions; "Excitation"— excitatory scaling interventions; "Spines"—dendritic spine assessment; "Channels"—quantification of synaptic channels; "Syn Prot"—quantification of other synaptic proteins; "Interference"—manipulations interfering with mechanisms of synaptic scaling; "Hebbian"—investigation of the effects of scaling on Hebbian plasticity. Displayed numbers are the ρ coefficients from Spearman's correlations, which are represented in bold if significantly correlated after Bonferroni correction for multiple comparisons.

the most adopted model in studies reporting excitatory manipulations ($\rho = 0.35$, $p < 0.0001$), when membrane channels were assessed ($\rho = 0.33$, $p < 0.0001$), and when there were interferences with synaptic scaling mechanisms ($\rho = 0.22$, $p = 0.004$). However, this model was avoided if the articles were investigating the relationship between Hebbian and synaptic scaling types of plasticity ($\rho = -0.25$, $p = 0.001$). Studies employing models of sensorial manipulations follow an opposite pattern, as they are less prevalent when articles report excitatory manipulations ($\rho = -0.25$, $p = 0.001$), or membrane channel measurements ($\rho = -0.21$, $p = 0.00$), but are preferred when Hebbian plasticity is considered ($\rho = 0.18$, $p = 0.006$). Finally, when studies induce synaptic plasticity by *in vivo* circuitry manipulations, we observe an increase in the report of mIPSCs evaluations ($\rho = 0.27$, $p = 0.0004$).

Risk of Bias Assessment

The description of measures to reduce risk of bias within each study was evaluated by reporting of the following items: blinded assessment of outcomes, unbiased data selection, sample size and/or power calculation, statement of compliance with regulatory requirements, statement of approval by an ethics committee, and statement on conflict of interest (see Methods for definitions of each item). We analyzed the frequency of reporting for each of these items, as well as its correlation with the publication year (**Table 3**). Our results are comparable with previous studies that described a low incidence of reporting risk of bias measures for animal disease models (Sena et al., 2014; Macleod et al., 2015), and for basic-research paradigms such as fear conditioning (Carneiro et al., 2018). In our sample, the reporting of common features, such as regulatory compliance statement, ethics committee approval, and conflict of interest, was observed in less than half of articles. However, these features showed a significant increase over time, suggesting that the increase of reporting demands, perhaps due to journal policies (McNutt, 2014), is having an impact on this field.

Next, we analyze the correlation between overall reporting score (i.e., the fraction of reported risk-of-bias measures) and year of publication or impact factor (**Figure 4**). Interestingly,

TABLE 3 | Risk of bias measures.

Attribute	# Articles (%) [95% C.I]	Trend over time (ρ)	*p*-value
Blinded outcome assessment	35 (20.8) [14.7, 27.0]	−0.118	0.129
Unbiased data selection	28 (16.7) [11.8, 23.0]	−0.020	0.792
Power or sample size calculation	4 (2.4) [0.9, 5.9]	0.166	0.032
Regulatory compliance statement	77 (45.8) [38.4, 53.3]	0.209	0.007#
Conflict of interest*	72 (42.9) [35.6, 50.4]	0.529	<0.0001#
Ethics committee approval**	74 (46.0) [38.4, 53.6]	0.387	<0.0001#

*The first column shows the number of articles reporting each item, with percentages relative to the total number of articles included (n = 168, except for ethics committee approval), and their 95% confidence intervals. Spearman's correlation was used to estimate the ρ coefficient and p-values for reporting trends over time. *Of these, 4 reported an existing conflict of interest. **Seven articles used invertebrate models, which usually do not require the approval of an ethics committee; therefore, the denominator for this item was 161. #Significantly correlated with time (α = 0.0083 after Bonferroni correction for multiple comparisons).*

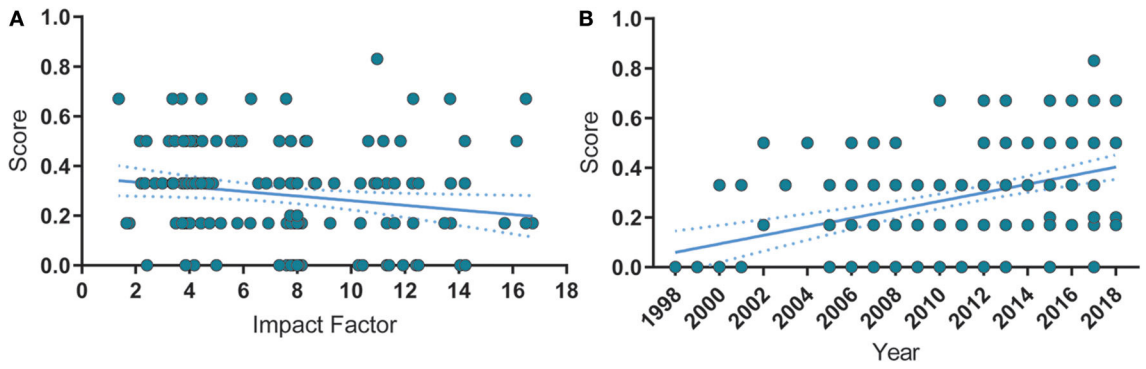

FIGURE 4 | Correlations between reporting score, publication year and impact factor. **(A)** Risk of bias reporting score is negatively correlated with the impact factor. Spearman's $\rho = -0.24$, $p = 0.002$. **(B)** Over time, the quality score significantly increased. Spearman's $\rho = 0.425$, $p < 0.0001$. Spearman's $\rho = -0.001$, $p = 0.904$. Solid lines represent the linear fit of the data. Dashed lines are the 95% C.I. of the linear fit.

the risk of bias reporting score correlated negatively with the impact factor (**Figure 4A**), although the overall reporting score improved over the years (**Figure 4B**), indicating that publication in high-impact journals does not safeguard the correct reporting of measures to prevent risk of bias.

DISCUSSION

The synaptic scaling literature is relatively recent, as the first experimental evidence for this phenomenon was described around 20 years ago (Turrigiano et al., 1998). Nevertheless, we observed a noteworthy number of studies on the subject since then, and a growth in publication volume over the last two decades. The impact factor of the publishing journals has been maintained over time, indicating sustained visibility on the topic. As the systematic reviews of basic-research literature are not usual, such characteristics provide a unique opportunity to compare methods and quality indicators of a relatively new area of basic research to more studied fields, especially applied pre-clinical research.

Our first observation is that the majority of articles in our sample use *in vitro* (83.3%), rather than *in vivo* (18.4%) models, which are mostly based on rodents (51.8% rats, 39.3% mice). This is somewhat expected, as there are many challenges for *in vivo* studies (Lee and Kirkwood, 2019), and *in vitro* experiments would allow for more convenient manipulations for chronic neuronal excitation or inhibition, such as a constant pharmacological administration or direct light stimulation for optogenetics. Accordingly, neuronal cultures were the most popular experimental model (58.9% dissociated cells, 18.4% organotypic). Interestingly, articles reporting experiments employing rats are negatively correlated with time ($\rho = 0.283$, $p = 0.0002$), while the usage of mice seems to be rising ($\rho = 0.225$, $p = 0.0034$). This can indicate a shift from the use of rats to mice models, possibly due to the development of genetic manipulations, which are more easily performed in mice (Fahey et al., 2013).

When examining the experimental features of the articles, we observe that most studies have investigated synaptic scaling after chronic inhibition of neuronal activity (86%). Moreover, reporting of inhibitory interventions to induce scaling has a negative correlation with the reporting of excitatory manipulations ($\rho = -0.48$, $p < 0.0001$). That is a somewhat counterintuitive preference, as the field has long stated the theoretical importance of homeostatic mechanisms for protecting network stability, usually from the effects of excessive activity caused by Hebbian types of plasticity (Abbott and Nelson, 2000; Turrigiano, 2012). Furthermore, the number of empirical studies in our sample about the effects of synaptic scaling on Hebbian-like processes was small (4%), showing that there is some dissonance between theoretical concerns and experimental directions. As many questions on the interaction of these different types of plasticity remain open (Keck et al., 2017b), further research on the topic is required.

The standard practice to demonstrate homeostatic changes is by measurements of parameters of synaptic transmission (i.e., the analysis of presynaptic neurotransmitter release frequency or postsynaptic response amplitude). Accordingly, more than 70% of the studies in our sample assessed synaptic scaling through miniature excitatory postsynaptic currents (mEPSCs), which has been used since the first article describing scaling. On the other hand, miniature inhibitory postsynaptic currents (mIPSCs) were investigated in less than 15% of the articles, which is rather scarce considering that both excitatory and inhibitory currents are thought to be regulated to reach homeostatic activity (Swanwick et al., 2006). Thus, we encourage the investigation of scaling-driven regulation of inhibitory currents in forthcoming studies of the field.

Synaptic scaling can also be explored by examining morphological or molecular markers, such as dendritic spines, synaptic receptors/ channels, and other activity-modulated synaptic proteins. We can observe a correlation in the reporting of measurements of synaptic channels and other synaptic proteins ($\rho = 0.27$, $p = 0.0004$), suggesting that

such morphological parameters are analyzed concomitantly. However, our review shows that these types of experiments are not performed as frequently as the miniature post-synaptic current assessments, indicating that the commonly-accepted demonstrations of scaling-induced changes might be restricted to electrophysiological measurements. We thus believe that the consolidation of alternative parameters, like molecular markers, to confirm the occurrence of synaptic scaling could broaden the experimental range of the field, as it would be more accessible for researchers with different technical expertise.

A large part of the studies uses protocols interfering with homeostatic processes (70%), i.e., using pharmacological or genetic manipulations of specific molecules or cascades to identify those involved in the synaptic scaling. In fact, the only temporal trend found within the experimental features was the growth in the number of such reports over time, suggesting that the field is increasingly focused on the mechanistic description of homeostatic regulation. Our association analysis also showed that these articles are more likely to report mEPSCs measurements ($\rho = 0.28$, $p = 0.0002$) and quantifications of synaptic channels/ receptors ($\rho = 0.29$, $p = 0.0001$). Nevertheless, a surprisingly smaller amount of articles investigated fundamental assumptions of synaptic scaling, like its functional role in firing rate homeostasis (24%) or the multiplicative nature of the synaptic changes (29%). Given that post-synaptic currents can be regulated in a non-homeostatic manner (Diering and Huganir, 2018) and that many other types of homeostatic mechanisms do not involve multiplicative adjustments (Keck et al., 2017a; Wang et al., 2019), such assessments are essential for proper identification of scaling-specific processes. Thus, we believe that further attention should be given to confirming the extent of basic scaling features alongside with the employment of homeostatic-inducing interventions.

Moreover, within the articles that mention the multiplicative nature of synaptic scaling, we assessed which ones actually performed statistical tests for its confirmation (**Supplementary Figure 1**). The most accepted method for determining whether or not multiplicative scaling occurred is based on the analysis of amplitude distributions of the miniature post-synaptic currents (Kim et al., 2012), which can also be applied for correspondent measurements of synaptic puncta, proteins or channels (Keck et al., 2013). First, the recorded amplitudes from the treated cells are rank-ordered and plotted against the rank-ordered control amplitudes. This plot is then fitted with a straight line to obtain the scaling function and, consequently, the scaling factor. Secondly, the individual amplitude values of treated neurons are multiplied by the scaling factor, and a cumulative frequency plot of these amplitudes is constructed. Lastly, the overlap between the treated and control recordings is compared by the Kolmogorov-Smirnov test. Among the 49 articles mentioning multiplicative scaling, 13 (26%) describe employing linear regression of the ranked amplitudes, 14 (29%) report the Kolmogorov-Smirnov comparison analysis of cumulative amplitude distributions, and 16 (33%) describe the whole method with both steps. Interestingly, 6 studies (12%) do not describe any approach for multiplicative scaling assessment, although mentioning this feature in the manuscript. These results indicate that most of articles have a good description of at least of the main steps for multiplicative scaling confirmation.

Regarding the quality of reporting, despite being mainly published in high profile journals, our sample had comparable performance to other areas of animal research in terms of describing procedures to reduce the risk of bias. Less than half of the articles reported basic information such as regulatory compliance statements, ethics committee approval, and conflict of interest. Reporting of blinded outcome assessment was even less frequent and present in only around 20% of the articles. The frequency of reporting for these items was lower than those found in a review of preclinical fields (Macleod et al., 2015) and in a systematic review on fear conditioning (Carneiro et al., 2018). Likewise, sample size or power calculations were performed in a negligible portion of the studies (2.4%).

In addition to these commonly used indicators, we assessed the description of measures to reduce bias in data selection (e.g., randomly selecting mEPSC recordings from a large set; blinding or automatizing the process of selecting images for analysis). To our knowledge, this feature has not been investigated in previous reviews, but as technological advances make it easier to collect large amounts of data on numerous types of experiments, we believe that explicit criteria to select data for analysis are a vital part of a study's methodology. This item was reported in 16.7% of articles in our sample, an encouraging result given the lack of discussion on this topic; however, a value that is still suboptimal for a field highly dependent on extensive data collection.

A commitment to improving *in vivo* research has been stated as a priority by many publishers (McNutt, 2014). Journal demands on conflict of interest disclosures and ethics statements seem to have influenced the synaptic scaling literature, as reporting of these features has significantly increased over time (**Table 1**). Interestingly, however, the impact factor of the journals is negatively correlated with our risk of bias reporting score in our sample. This diverged from previous reports that have found no statistically significant correlations between these attributes (Macleod et al., 2015; Carneiro et al., 2018). Nonetheless, our sample had a higher median impact factor than the ones analyzed in other reviews, which might indicate that this relation can only be observed in restricted parts of the journal impact factor distribution.

One can argue that high-impact journals have historically imposed strict word count limits, which might have negatively impacted reporting. However, the more recent availability of nearly limitless supplementary data online makes this explanation less likely. There is also evidence that reporting checklists used by high-visibility journals may be less effective than desired: a study that investigated whether journal-requested completion of an ARRIVE checklist improved compliance with the guidelines found little evidence of effectiveness (Hair et al.,

2019). Further investigations on the efficiency of journal policies to improve reporting are warranted to broaden this discussion.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: http://doi.org/10.7303/syn21165370.

AUTHOR CONTRIBUTIONS

TM conceived and designed the systematic review, prepared the figures, and wrote the manuscript. TM and DR extracted data and performed the statistical analysis. MW and HS contributed with supervision, manuscript editing, and funding acquisition. All authors revised the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fncel.2020.00164/full#supplementary-material

Supplementary Figure 1 | Types of analysis for multiplicative scaling assessment. From the 168-studies sample, 49 (29%) mention observing multiplicative scaling of mPSC amplitudes. Within articles investigating multiplicative scaling, 14 (28.6%) describe using the Kolmogorov-Smirnov test for comparison of cumulative amplitude distributions, 13 (26.5%) employ linear regression of the ranked amplitudes, and 16 (32.6%) report using both approaches. A number of studies (23) mentioned the use of the Kolmogorov-Smirnov test without assessment of multiplicative scaling, but for simple group-comparison analysis.

Supplementary Table 1 | Country affiliation of the corresponding author. Percentages were calculated based on the total number of articles ($n = 168$). If an author had more than one country affiliation, the article counted for both of them; thus, the sum of percentages exceeds 100%.

Supplementary Table 2 | Main manipulations to induce synaptic scaling. The table shows the number of articles reporting the use of a given intervention, the percentages relative to the total number of articles included ($n = 168$), and their 95% confidence intervals. Spearman's correlation was used to estimate the ρ coefficient and p values for reporting trends over time. $^{\#}$Significantly correlated with time ($\alpha = 0.005$ after Bonferroni correction for multiple comparisons).

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Homeostatic Plasticity in Epilepsy

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In the healthy brain, neuronal excitability and synaptic strength are homeostatically regulated to keep neuronal network activity within physiological boundaries. Epilepsy is characterized by episodes of highly synchronized firing across in widespread neuronal populations, due to a failure in regulation of network activity. Here we consider epilepsy as a failure of homeostatic plasticity or as a maladaptive response to perturbations in the activity. How homeostatic compensation is involved in epileptogenic processes or in the chronic phase of epilepsy, is still debated. Although several theories have been proposed, there is relatively little experimental evidence to evaluate them. In this perspective, we will discuss recent results that shed light on the potential role of homeostatic plasticity in epilepsy. First, we will present some recent insights on how homeostatic compensations are probably active before and during epileptogenesis and how their actions are temporally regulated and closely dependent on the progression of pathology. Then, we will consider the dual role of transcriptional regulation during epileptogenesis, and finally, we will underline the importance of homeostatic plasticity in the context of therapeutic interventions for epilepsy. While classic pharmacological interventions may be counteracted by the epileptic brain to maintain its potentially dysfunctional set point, novel therapeutic approaches may provide the neuronal network with the tools necessary to restore its physiological balance.

Keywords: homeostatic plasticity, epilepsy, excitation inhibition balance, gene therapy, synaptic transmission, REST (RE-1 silencing transcription factor)

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INTRODUCTION

Epilepsy is a heterogeneous group of complex diseases, with intricate temporal profiles. In many common forms such as temporal lobe epilepsy associated with hippocampal sclerosis, the brain undergoes a process of epileptogenesis, culminating in the symptomatic, chronic phase, characterized by interictal discharges and overt seizures (Devinsky et al., 2018). It stands to reason that the cellular and molecular processes linked to the development of epilepsy would follow an equally complex temporal profile. Similarly, the brain's intrinsic mechanisms to counter the detrimental effects caused by epilepsy are likely to be differentially regulated in epileptogenesis and the chronic epileptic phase. A simplistic interpretation of epileptogenesis is that it is a process that results in an imbalance of excitation and inhibition. However, a more complete understanding of epilepsy requires the inclusion of multiple dimensions, e.g., anatomy, synaptic and cellular features, transcriptome, and circuits dynamics. These dimensions in the phase space of the brain may have very different temporal dynamics and are, given biological constraints, often non-orthogonal. The healthy

brain is a dynamic system that operates, most of the time, within certain boundaries in its physiological multidimensional zone while epileptogenic factors pull its trajectories towards pathological regions. In epilepsy, the brain crosses these boundaries more often, eventually resulting in seizures, so it can be defined as a continuous interchange between epileptic/pathological and physiological brain states associated with the occurrence of epileptic activity (Abreu et al., 2019). The physiological mechanisms that can confine the brain's state inside healthy phase-space boundaries, despite epileptogenic attractors, fall squarely within the definition of homeostatic plasticity (Turrigiano, 2012). While many examples of homeostatic downscaling in the face of disinhibition or overexcitation can be observed *in vitro* or *ex vivo* (Grubb and Burrone, 2010; Sun and Turrigiano, 2011; Barnes et al., 2017; Xu and Pozzo-Miller, 2017; Chowdhury et al., 2018), hyperexcitability-induced homeostatic plasticity is a relatively less characterized phenomenon in complex systems *in vivo* (Lee and Kirkwood, 2019). In particular, the role of the homeostatic machinery once chronic epilepsy has been established is still unknown. In principle, in a persistently hyperexcitable network, homeostatic mechanisms should bring the brain state back to a physiological space, but this is not what has been observed in rodent models and human patients (André et al., 2018). One of the characteristic features of an epileptic brain is an aberrant recurrent hyperactivity not present in non-pathological circuits (Chang and Lowenstein, 2003). Therefore, by definition, an epileptic brain is one in which homeostatic plasticity fails to maintain the network's physiological boundaries. Several alterations, probably alongside compensations, occur during the epileptogenesis period leading to hyperexcitable circuits which cannot be compensated by homeostatic plasticity, leaving the brain in an abnormal state and eventually causing seizures. The shift to a pathological state can be also related to the transition between interictal and ictal activity (Khambhati et al., 2017). A possibility is that pathogenic events shift the homeostatic equilibrium closer to the transition point between interictal and ictal states, effectively making the plastic changes maladaptive. While plausible, this hypothesis is difficult to test in a highly dynamical system where the homeostatic set "point" is constantly shifting in response to Hebbian and homeostatic perturbations.

Why Is Homeostatic Plasticity Unable to Suppress Seizures in Epilepsy?

The precise mechanisms by which seizures arise are still debated, but it is widely speculated that some circuits become overactive (Devinsky et al., 2018). Why homeostatic plasticity is not able to counteract this aberrant network activity is still unknown. One possibility, plausible in acquired epilepsies, is that a gradual weakening of the homeostatic response or a maladaptive compensation may be due to the progressive neuronal degeneration during epileptogenesis. The loss of a small percentage of interneurons may have huge consequences in the network's ability to maintain the brain in its physiological space (Houweling et al., 2005; Cossart, 2014; Queenan et al., 2018). Another possibility, that would better explain genetic epilepsies, is that the homeostatic processes occurring during the epileptogenesis, e.g., compensation of a mutated gene function, maybe at the basis of the hyperactive network observed in the chronic phase, because of the impossibility of a biological system to constantly compensate for the chronic loss of key proteins fundamental to maintain the brain within physiological boundaries.

In both cases, the failure of homeostatic plasticity in suppressing network hyperexcitability may be attributed to a failure of cellular and/or molecular mechanisms that would normally re-establish and constantly maintain the network's physiological boundaries.

TEMPORAL PROFILE OF HOMEOSTATIC ADAPTATIONS IN EPILEPSY

Neuronal networks are highly dynamic systems that require appropriate compensation. Homeostatic plasticity can act on a variety of different sub-cellular signaling cascades to regulate activity (Wefelmeyer et al., 2016). Similarly, the temporal profile of homeostatic plasticity must evolve to follow the network's requirements for regulation with minimal disruption of its function. An example observed in non-pathological conditions is the developmental change in the synaptic valence of GABAergic transmission. While in the mature brain opening of GABAAR commonly leads to an influx of chloride ions and subsequent hyperpolarization, in early development chloride concentration is higher in the cell leading to GABAAR-mediated depolarization due to the efflux of chloride ions. A recent study of a specific type of interneurons, Chandelier cells, shows that, in response to sustained stimulation, GABAergic inputs are homeostatically reduced in early developmental stages (high intracellular chloride) and increased in the mature system when GABA has an inhibitory effect (Pan-Vazquez et al., 2020). This example underlines how homeostatic processes are developmentally regulated and that the direction of the compensations is dynamically guided by the network state rather than be simply fixed at the single-cell level. Experimental models of epilepsy offer a unique opportunity to study the evolution, and failures, of homeostatic processes. Most of these models introduce a perturbation in the system that leads to expanding the boundaries of the brain's trajectory towards seizure space. In some cases, these extensions in phase space trajectory outlast the duration of the perturbation, as in the case of intracranial infusion of Tetanus toxin (TeNT). The TeNT is a small protein that impairs preferentially GABAergic release by cleaving Synaptobrevin2 at interneuron terminals (Schiavo et al., 2000). Intracranial injections of TeNT are used to induce epilepsy with two distinct phases: (I) an acute phase with a high number of ictal events and detectable TeNT activity; and (II) a chronic phase with no TeNT activity and a slowly decreasing number of ictal events (Jefferys et al., 1995; Mainardi et al., 2012; Wykes et al., 2012; Vannini et al., 2016; Chang et al., 2018; Snowball et al., 2019). In this model, the impairment of a key "homeostatic tool" prevents the system from reaching its physiological set point. However, looking at the ultrastructural level, homeostatic mechanisms are still put in place to reduce

the network's hyperexcitability. In mice injected with TeNT in the visual cortex, the active zone length of inhibitory synapses is significantly increased in the acute phase of TeNT-induced focal epilepsy (Vannini et al., 2020). At this stage. increase in active zone size is unlikely on its own to have a major effect on GABA release, given the continued catalytic effect of TeNT at this stage of the model. At a later point, changes occur in the organization of the functional fraction of excitatory vesicles. Synaptic release in response to mild visual stimulation is similar in control and epileptic mice but vesicular positioning within the presynaptic terminal is considerably different. While in control conditions release-competent vesicles are spatially biased toward the active zone (Marra et al., 2012; Rey et al., 2015), in the chronic phase of TeNT-induced epilepsy functional vesicles are evenly distributed within the cluster, presumably reducing synchronization of excitatory vesicles' release (Vannini et al., 2020). It is plausible that increasing the average distance between functional vesicles and release site has an impact on temporal and filtering properties of excitatory synapses, changing the synaptic transfer function (action potential to vesicular release) so that high-frequency firing (typical of seizure) has a lower output while leaving information transmission within healthy space relatively unchanged (Trigo et al., 2012; Pulido et al., 2015; Pulido and Marty, 2017; Miki et al., 2018). The change in the positioning of release-competent vesicles is preceded by a sustained increase in Carboxypeptidase E, a protein required for vesicle positioning in the proximity of their release site (Park et al., 2008; Lou et al., 2010; Vannini et al., 2020). This adaptation of homeostatic mechanisms over time, and across different neuronal types, is an example of the complex and dynamic processes involved in maintaining neuronal function within healthy boundaries by acting on seemingly independent synaptic features. However, if the time course of the homeostatic response does not match closely the one of its triggering cause, plasticity may lead to a maladaptive regulation of network activity. For example, ischemic events or traumas may transiently impair neurotransmission and as a result, the system will compensate for reduced excitatory inputs becoming less stable and more likely leading to an ictal state.

RE-1 SILENCING TRANSCRIPTION FACTOR's (REST's) JANUS ROLE IN HOMEOSTATIC PLASTICITY/EPILEPTOGENESIS

The temporal dynamic adaptation of homeostatic processes during the epileptic phases is also reflected at the transcriptomic level by differential changes in the regulation of gene expression. Indeed, growing evidence demonstrates that the same homeostatic transcriptomic pathways which in some conditions favor the recovery of a physiological set point, in other conditions exert the opposite action exacerbating neuronal hyperactivity (Baldelli and Meldolesi, 2015).

A clear example is offered by the debated role of RE-1 Silencing Transcription Factor (REST), also known as neuron-specific silencing factor (NRSF), in homeostatic plasticity. This gene-silencing transcription factor, widely expressed during embryogenesis, exerts a strategic role (Ballas et al., 2001; Roopra et al., 2001; Ooi and Wood, 2007) during the late stages of neuronal differentiation when the loss of REST is critical for the acquisition of the neuronal phenotype (Su et al., 2006).

In mature neurons, REST exhibits several unique properties. Indeed, its expression is increased by kainate-induced seizures *in vivo* (Palm et al., 1998; Gillies et al., 2009) and chronic hyperactivity in cultured neuronal cultures (Pozzi et al., 2013). Interestingly, REST induces firing homeostasis by downregulating voltage-gated Na^+ channel expression in excitatory neurons (Pozzi et al., 2013) and scales down the strength of excitatory synapses, acting presynaptically, in response to chronic hyperactivity (Pecoraro-Bisogni et al., 2018). Because REST knockdown impairs both intrinsic and synaptic homeostasis, these results indicate that REST function is critical for inducing homeostatic negative feedback responses to readjust the network firing activity at a physiological set point and protect it from hyperactivity. Following this homeostatic role, a 2-deoxy-D-glucose ketogenic diet was reported to have an antiepileptic effect *via* the activation of a chromatin remodeling complex controlled by an increase in REST (Garriga-Canut et al., 2006) and, in the kindling model of epileptogenesis, conditional REST deletion in excitatory neurons of the postnatal mouse forebrain resulted in a dramatic acceleration of seizure progression and prolonged after-discharge duration compared with control mice (Hu et al., 2011).

On the other hand, in the kainate mouse model of temporal lobe epilepsy, blocking REST function repressed the expression of the hyperpolarization-activated, cyclic nucleotide-gated channel (HCN1) attenuating the epileptic phenotype (McClelland et al., 2011). Subsequently, the same authors revealed that the repression resulting from REST increase was not limited to HCN1 but also included 10% of the analyzed target genes. REST inhibition was found to lead to attenuation of seizures, strongly supporting the hypothesis that seizure-induced increases of REST contribute to epileptogenesis *via* REST-mediated repression of a group of genes that critically influence neuronal function (McClelland et al., 2014).

These contrasting effects still prevent us from concluding whether inhibition or enhancement of REST signaling should prevent epileptogenesis. To address this question, it will be essential to evaluate how REST changes its influences depending on conditions, for example, cell specificity, neural networks, expression timing and loci, and status of progression of epilepsy. However, a reading key that could permit to better interpret why REST-signaling is in some cases homeostatic while exerts in other cases an opposite pro-epileptogenic action, is to consider that probably the primary tasks of homeostatic plasticity is aimed to constitute a constrain at the saturation of use-dependent Hebbian plasticity (Turrigiano, 2012; Li et al., 2019).

Therefore, the homeostatic efficacy of REST is possibly effective in the initial stages of epileptogenesis, when the level of hyperactivity has not yet turned away the neuronal network too far from its physiological space. Indeed, considering that REST-signaling is strictly dependent on the neuronal hyperactivity, when this assumes excessive values, due to

chronic pathological conditions, the complete dysregulation of the REST-pathway could transform its homeostatic capacity in a pro-epileptogenic function, thus contributing to the consolidation and aggravation of chronic epilepsy.

Nevertheless, homeostatic plasticity can still be considered as a therapeutic target for the protection from epilepsy even in chronic epileptic conditions, thanks to the possibility of subtracting homeostatic plasticity from the control of advanced pathological hyperactivity, through its direct and exogenous modulation by pharmacological and genetic strategies.

THE COMPLEX UNIQUE PROPERTIES OF THE HYPERPOLARIZATION-ACTIVATED CATION CURRENT (Ih) IN HOMEOSTATIC PLASTICITY AND EPILEPSY

In many cases, the same molecular actors playing crucial roles in homeostatic plasticity are, in a different time or place, fundamental mediators of the epileptogenic processes. A paradigmatic example of such complex interpenetration between homeostatic plasticity and epileptogenesis is offered by the Ih, that in recent years was ascribed as a central player of both homeostatic and epileptogenic processes. Ih is mixed sodium and potassium conductance generated by the hyperpolarization-activated cyclic nucleotide-gated (HCN) channels and activated by membrane hyperpolarization. Initially discovered in the pacemaker heart sinoatrial node cells and subsequently found to be widely expressed in the central and peripheral nervous system (Brown et al., 1979; Robinson and Siegelbaum, 2003). Ih plays an important role in determining membrane potential and firing characteristics of neurons and therefore is a potential target for homeostatic regulation. Indeed, in CA1 pyramidal cells, Ih was found to be up- or down-regulated following chronic (48 h) hyperactivity or activity deprivation, respectively. Such bidirectional homeostatic regulation not only controls spiking activity but also stabilizes the threshold for long-term potentiation induced in CA1 pyramidal neurons by repetitive stimulation, accelerating EPSP kinetics, and reducing temporal summation of EPSPs (Gasselin et al., 2015). These results suggest that modulation of Ih represents a homeostatic plasticity mechanism, allowing neurons to control their excitability and EPSP summation in response to changes in synaptic activity on both short and long-term time scale. Furthermore, the earliest reports of HCN channel dysfunction in epilepsy revealed the enhancement of somatic Ih in hippocampal CA1 pyramidal neurons of animal models of febrile seizures, and more recently, in a mouse model of fragile X with audiogenic seizures (Chen et al., 2001; Bender et al., 2003; Dyhrfjeld-Johnsen et al., 2008; Brager et al., 2012). These results were unexpected, as an increase in Ih is considered to be inhibitory, questioning that these changes may be epileptogenic and suggesting that they can potentially reflect a homeostatic process in response to augmented neural network activity.

However, in contrast with the above-mentioned results, in most experimental paradigms for investigation of recurrent epileptic seizures following administration of convulsant agents, a reduction of Ih was observed in multiple cortical and hippocampal regions (Shah et al., 2004; Jung et al., 2007; Marcelin et al., 2009). Furthermore, the deletion of HCN1 in mice resulted in greater seizure susceptibility (Huang et al., 2009; Santoro et al., 2010) and loss of function mutations in HCN1 have been recently reported causing severe neonatal epileptic encephalopathies (Marini et al., 2018).

In summary, these data can be explained only considering the complex and dynamic role exerted by the modulation of HCN channels in both epilepsy and homeostatic plasticity. The timing is crucial, with different regulation of HCN1 in the epileptic phases, with a decrease in expression during epileptogenesis followed by an increase, potentially homeostatic, in the chronic phase. On the other hand, HCN1 localization, indirect action, and overall transcriptome could influence its function. A decrease in HCN channels expression will hyperpolarize the resting membrane potential (RMP) inhibiting neuronal excitability, but at the same time, it will increase the membrane input resistance (R_{in}), exerting an excitatory action, because of the reduction of the amount of current needed to depolarize the cell (Kase and Imoto, 2012). Moreover, HCN channels are differentially expressed across the brain and neuronal populations, showing also a heterogeneous subcellular distribution, with high expression in the dendrite and lower expression in the soma (Magee, 1999). Importantly Ih net effect on excitability depends on the cell-specific interplay of passive and active membrane conductance. Indeed, multiple reports have shown that Ih currents affect particularly the activity of other co-expressed subthreshold conductances (George et al., 2009; Amarillo et al., 2014; Hu and Bean, 2018). The final effect of HCN modulation therefore will depend on the specific combination of such subthreshold conductances that change in different neuronal populations and consequently the net outcome of a similar Ih modulation can be a reduced excitability in some cases and increased excitability in others.

The HCN example highlights how the role of homeostatic plasticity in epilepsy is complex and dynamic, depending not simply on temporal and spatial expression of a gene and its protein, but also the interactions between differently expressed proteins and thus on the overall transcriptome and proteasome.

THERAPEUTIC INTERVENTIONS BASED ON THE "GENETIC LEAD" OF HOMEOSTATIC COMPENSATIONS

Gene therapy for epilepsy is a promising approach to treat the chronic phase of the pathology (Kullmann et al., 2014). Recent gene therapies target the symptoms (seizures) rather than the cause of epilepsy, for example, decreasing the excitability of excitatory neurons or potentiating inhibitory tone (Richichi et al., 2004; Noè et al., 2008; Wykes et al., 2012; Krook-Magnuson et al., 2013; Kätzel et al., 2014; Lieb et al., 2018; Agostinho et al., 2019; Wickham et al., 2019; Colasante et al., 2020). These therapies have been efficient in decreasing intrinsic neuronal excitability, synaptic transmission, and the number of seizures, in rescuing cognitive defects and also

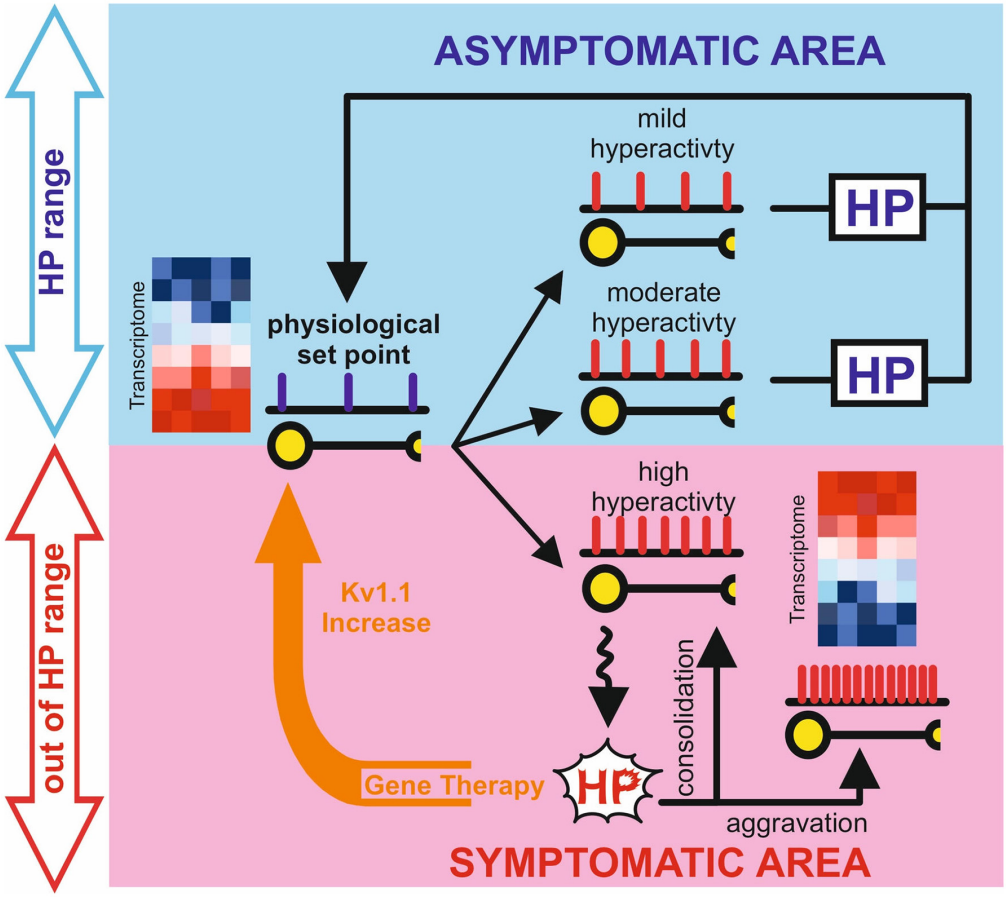

FIGURE 1 | Graphical representation of the potential role of homeostatic plasticity in the epileptic process. The blue box represents the physiological space (Asymptomatic Area) in which homeostatic plasticity, operating within the limits of physiological condition, is still able to counteract changes in neuronal activity to reset the correct setpoint. The light red box represents the pathological space (Symptomatic Area), in which homeostatic plasticity has failed or even aggravate the network state. Gene therapy interventions (orange arrow) can reset the physiological set point in the chronic phase of epilepsy probably "boosting" the inefficient homeostatic plasticity. Representative heatmaps represent gene expression in physiological and pathological conditions, where blue is gene downregulation and red is gene upregulation.

in resetting a physiological transcriptomic profile. In these cases, no homeostatic compensations have been observed to counteract the decreased excitability induced by the therapeutic approach. Furthermore, a net positive effect at transcriptomic level induced by an increase of endogenous Kv1.1 using CRISPRa, suggests a compensatory mechanism in line with a response to an increased network activity (Colasante et al., 2020). This effect was surprising because of the uncertainty on the effect of gene therapy: does it only increase seizure threshold or does it also rescue the epileptogenic process pushing back the brain state within its physiological boundaries? Showing cognitive deficits and gene expression rescue, the data pointed towards the latter. Importantly, Kv1.1 has been recently associated with *in vivo* homeostatic response, where to compensate network hyperexcitability, neurons increased endogenous Kv1.1 expression leading to a clear reduction of their firing rate (Morgan et al., 2019). These data together provide new insights, but also open a new series of questions, on the possible homeostatic mechanisms occurring in chronic established epilepsy. It indicates that the brain is in a chronic altered state in which homeostatic plasticity cannot maintain activity in its physiological boundaries, but that with a "homeostatic boost" in the direction of a reduction in neuronal excitability, the entire network may be able to rearrange itself to a less excitable state and take back control of phase-space regulation. Because Kv1.1 is implicated in fast homeostatic response *in vivo*, its increase could potentially drive the network compensation observed (Morgan et al., 2019). Indeed, the potential homeostatic role of Kv1.1 expression has been also corroborated in other experimental settings. It has been shown that Kv1.1 reduction improves spike timing precision and thus synchronization, therefore an increase in Kv1.1 expression could desynchronize the hypersynchronous epileptic network and in this way decrease network activity (Cudmore et al., 2010). An example is an increase of Kv1.1 in Dentate Gyrus in a mouse model of TLE as a result of positive compensations to delay AP and decrease neuronal excitability (Kirchheim et al., 2013). Furthermore, Kv1.1 expression is tightly correlated with the expression of other

potassium channels, such as Kv7. Indeed, a homeostatic switch from Kv1.1 to Kv7.2 in the AIS after input deprivation in the avian cochlear nucleus has been shown to increase neuronal excitability, and on the other hand, hyperexcitation induced by Kv7 inhibition results in a fast intrinsic homeostatic response in line with a possible Kv1.1 increase in the AIS (Kuba et al., 2015; Lezmy et al., 2020). These data provide important pieces of evidence of the pivotal role of Kv1.1 in the homeostatic process and how its enhancement could lead to a remodeling of the pathological hyperexcitable network in epilepsy.

However is also possible that the Kv1.1 enhancement just increased the threshold for seizure generation (unlikely because not observed in acute seizure induction), that in turns allow a rearrangement of network activity with a consequent resetting of the transcriptional profile and physiological brain state (Colasante et al., 2020). Another possible explanation is that decreasing neuronal excitability to a certain extent, the system can be pushed back to a more stable interictal state, less likely to fluctuate go back into the ictal state. This hypothesis needs to be tested experimentally with depth electrode recordings in the epileptic focus, to capture the interictal activity before and after a gene therapy treatment.

Therefore, further experiments need to be performed to understand more in-depth this phenomenon and most importantly the duration of these compensatory effects. Furthermore, regarding genetic epilepsies, gene therapy interventions at a later stage could shed light on the rules underlying circuit rearrangement during epileptogenesis (Wykes and Lignani, 2018).

Finally, these new observations also suggest that the epileptic network is not reset at a different firing rate level but is in an unstable pathological space in which network compensation can still occur if driven by external interventions. This phenomenon underlies the importance of developing potential treatments for epilepsy based on the "genetic load" of the homeostatic plasticity mechanisms.

CONCLUSION

The role of homeostatic plasticity in Epilepsy is still not fully understood, however, new insights underline its importance in the temporal and dynamic dysregulation of the neuronal network in the consolidation of this pathology. Probably many unseen homeostatic compensations occur to protect the network for being hyperactive and prevent epileptogenesis and seizures. These physiological protective processes are difficult to observe experimentally and a full understanding of the molecular mechanisms underlying seizure-induced homeostasis without the confounding of epileptogenic processes is required. Relatively simple *in vitro* systems (e.g., dissociated or slice cultures), where homeostatic plasticity works in relative isolation, maybe better experimental models for the dissection of different homeostatic mechanisms. However, each finding *in vitro* will need to be validated in the higher dimensional phase space offered by *in vivo* in models of chronic epilepsy that preferably avoids neuronal injury and death, as the discrimination between injury-induced changes and homeostatic-induced mechanisms will be critical.

Given the wide variability of expression profiles that can lead to phenotypically undistinguishable physiological outputs (Marder and Goaillard, 2006), it is likely that slightly different epileptogenic histories may lead to engaging different and seemingly independent, homeostatic mechanisms. When the system's state crosses the limit within which homeostatic plasticity operates (**Figure 1**), these compensations cannot counteract neuronal hyperexcitability leading to a maladaptive or partial compensation. Therefore, further experiments need to be performed to clarify the role of homeostatic plasticity in epilepsy. These studies are likely to require modern experimental tools to dissect the fine physiological adaptation able to prevent seizure onset as well as define their operational boundaries.

Effective gene therapies that not only decrease seizures but also rescue gene expression need to be studied in more detail to understand the cascade of events that eventually lead to restoring a physiological network. A deeper understanding of successful homeostatic compensation of pathological brain state may lead to new targets for efficient therapeutic interventions, able to work in concert with the brain's physiological regulation of neural activity.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

All authors contributed equally to the manuscript.

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frontiers in Cellular Neuroscience

BRIEF RESEARCH REPORT
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Distinct Target-Specific Mechanisms Homeostatically Stabilize Transmission at Pre- and Post-synaptic Compartments

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Neurons must establish and stabilize connections made with diverse targets, each with distinct demands and functional characteristics. At *Drosophila* neuromuscular junctions (NMJs), synaptic strength remains stable in a manipulation that simultaneously induces hypo-innervation on one target and hyper-innervation on the other. However, the expression mechanisms that achieve this exquisite target-specific homeostatic control remain enigmatic. Here, we identify the distinct target-specific homeostatic expression mechanisms. On the hypo-innervated target, an increase in postsynaptic glutamate receptor (GluR) abundance is sufficient to compensate for reduced innervation, without any apparent presynaptic adaptations. In contrast, a target-specific reduction in presynaptic neurotransmitter release probability is reflected by a decrease in active zone components restricted to terminals of hyper-innervated targets. Finally, loss of postsynaptic GluRs on one target induces a compartmentalized, homeostatic enhancement of presynaptic neurotransmitter release called presynaptic homeostatic potentiation (PHP) that can be precisely balanced with the adaptations required for both hypo- and hyper-innervation to maintain stable synaptic strength. Thus, distinct anterograde and retrograde signaling systems operate at pre- and post-synaptic compartments to enable target-specific, homeostatic control of neurotransmission.

Keywords: active zone, homeostasis, synaptic plasticity, *Drosophila*, neuromuscular junction

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INTRODUCTION

Synapses are spectacularly diverse in their morphology, architecture, and functional characteristics. These differences are reflected in the molecular composition and abundance of synaptic components at heterogeneous synaptic subtypes in central and peripheral nervous systems (Atwood and Karunanithi, 2002; Branco and Staras, 2009; O'Rourke et al., 2012). Interestingly, the structure and function of synapses can also vary substantially across terminals of an individual neuron (Guerrero et al., 2005; Grillo et al., 2018; Fekete et al., 2019) and drive input-specific presynaptic plasticity (Letellier et al., 2019). Both Hebbian and homeostatic plasticity mechanisms can work locally and globally at specific synapses to tune synapse function, enabling stable yet flexible ranges of synaptic strength (Turrigiano, 2012; Vitureira and Goda, 2013; Diering and Huganir, 2018). For example, homeostatic receptor scaling globally adjusts glutamate receptor (GluR)

abundance, subtype, and/or functionality at dendrites (Turrigiano and Nelson, 2004) yet there is also evidence for synapse specificity (Sutton et al., 2006; Hou et al., 2008; Béïque et al., 2011). Although studies have begun to elucidate the factors that enable both local and global modes of synaptic plasticity at synaptic compartments, it is less appreciated how and why specific synapses undergo plasticity within the context and needs of information transfer in a neural circuit.

One major force that sculpts the heterogeneity of synaptic strength is imposed through the specific targets being innervated. For example, studies at neuromuscular synapses in the stomatogastric system of lobsters have demonstrated that presynaptic terminals of the same motor axon can concurrently undergo facilitation and depression due to differences in the synapses made onto two postsynaptic muscle fibers (Katz et al., 1993). Furthermore, at vertebrate neuromuscular junctions (NMJs), secreted factors from muscles can dictate which motor neurons survive during development and in many cases their neurotransmitter phenotype (Schotzinger and Landis, 1990; Calderó et al., 1998). Parallel target-dependent control of neuropeptide identity has also been shown in the *Drosophila* central nervous system (Allan et al., 2003; Allan and Thor, 2015). In mammalian central neurons, factors such as BDNF secreted from postsynaptic dendrites not only promote neuronal survival but also can homeostatically enhance presynaptic neurotransmitter release and functional properties of neural circuits (Jakawich et al., 2010; Park and Poo, 2013), while postsynaptic signaling through N-Cadherins and mTORC1 can regulate presynaptic function (Vitureira et al., 2011; Henry et al., 2012). Finally, at the *Drosophila* NMJ, presynaptic homeostatic plasticity can be expressed at a subset of terminals within a single motor neuron depending on GluR functionality at particular targets (Li et al., 2018a), demonstrating that this form of homeostatic plasticity is target-specific and strongly suggesting it is also synapse-specific. Together, these studies and others have demonstrated that the physiologic, metabolic, and/or structural properties at terminals of a single neuron can be selectively modulated according to the identity and needs of the targets they innervate. However, the nature of the trans-synaptic dialogue and the molecular mechanisms that achieve target-specific plasticity are not well understood.

A seminal study published over 20 years ago found that distinct target-specific modulations in synaptic activity maintain stable neurotransmission following biased innervation at terminals of motor neurons at the *Drosophila* NMJ (Davis and Goodman, 1998). In this manipulation, biased innervation is achieved by overexpression of the trans-synaptic cell adhesion factor *Fasciculin II* (*FasII*) on one of the two muscle targets innervated by motor neurons (Davis and Goodman, 1998). This leads to hyper-innervation of the target overexpressing *FasII* at the expense of the adjacent target, which is hypo-innervated. Remarkably, synaptic strength, as assessed by electrophysiological recordings, was maintained at levels similar in amplitude to normally innervated NMJ targets. Since this pioneering study, however, the molecular and cellular expression mechanisms that achieve this target-specific homeostatic modulation have remained enigmatic.

We have investigated how terminals of an individual neuron adapt to simultaneous hypo- and hyper-innervation to maintain stable synaptic strength on two adjacent targets. Our analysis reveals that a novel homeostatic signaling system operates in the hypo-innervated target to precisely enhance the abundance of postsynaptic GluRs, offsetting reduced presynaptic neurotransmitter release and stabilizing synaptic strength. In contrast, no apparent adaptations are observed in the hyper-innervated target. Rather, presynaptic release probability is homeostatically reduced, accompanied by a target-specific decrease in the abundance and density of active zone components. Finally, we find that presynaptic homeostatic potentiation (PHP) can be selectively induced and expressed at synapses on one target and balanced with biased innervation to sustain stable synaptic strength. This work reveals the striking interplay of target-specific homeostasis modulating the efficacy of neurotransmission across synaptic terminals.

MATERIALS AND METHODS

Fly Stocks

Drosophila stocks were raised at 25°C on standard molasses food. The w^{1118} strain is used as the wild type control unless otherwise noted as this is the genetic background in which all genotypes are bred. The *H94-Gal4* driver, which expresses transiently early in larval development (Davis et al., 1997), was sufficient to induce biased innervation when crossed to *UAS-FasII* (Davis and Goodman, 1998; used in **Figures 1–3**). However, this driver alone is not sufficient to knock-down *GluRIIA* when crossed to *UAS-GluRIIA-RNAi* (Li et al., 2018a). Therefore, the same manipulation developed in (Li et al., 2018a) was used for the experiments detailed in **Figure 4** and **Supplementary Figure S3**, where a cassette amplifies and maintains *Gal4* expression after transient activation by the *H94-Gal4* driver. This results in a persistently strong expression of the *UAS-FasII* and *UAS-GluRIIA-RNAi* transgenes in muscle 6. Details of all stocks and their sources are listed in the Reagents and Resource **Supplementary Table S1**.

Immunocytochemistry

Third-instar larvae were dissected in ice-cold 0 Ca^{2+} HL-3 and immunostained using a standard protocol as described (Perry et al., 2017). In brief, larvae were either fixed in Bouin's fixative for 5 min (Sigma, HT10132-1L), 100% ice-cold ethanol for 5 min, or 4% paraformaldehyde (PFA) for 10 min. Larvae were then washed with PBS containing 0.1% Triton X-100 (PBST) for 30 min, blocked with 5% Normal Donkey Serum followed by overnight incubation in primary antibodies at 4°C. Preparations were then washed 3× in PBST, incubated in secondary antibodies for 2 h, washed 3× in PBST, and equilibrated in 70% glycerol. Before imaging, samples were mounted in VectaShield (Vector Laboratories). Details of all antibodies, their source, dilution, and references are listed in **Supplementary Table S1**.

Confocal Imaging and Analysis

Samples were imaged using a Nikon A1R Resonant Scanning Confocal microscope equipped with NIS Elements software and

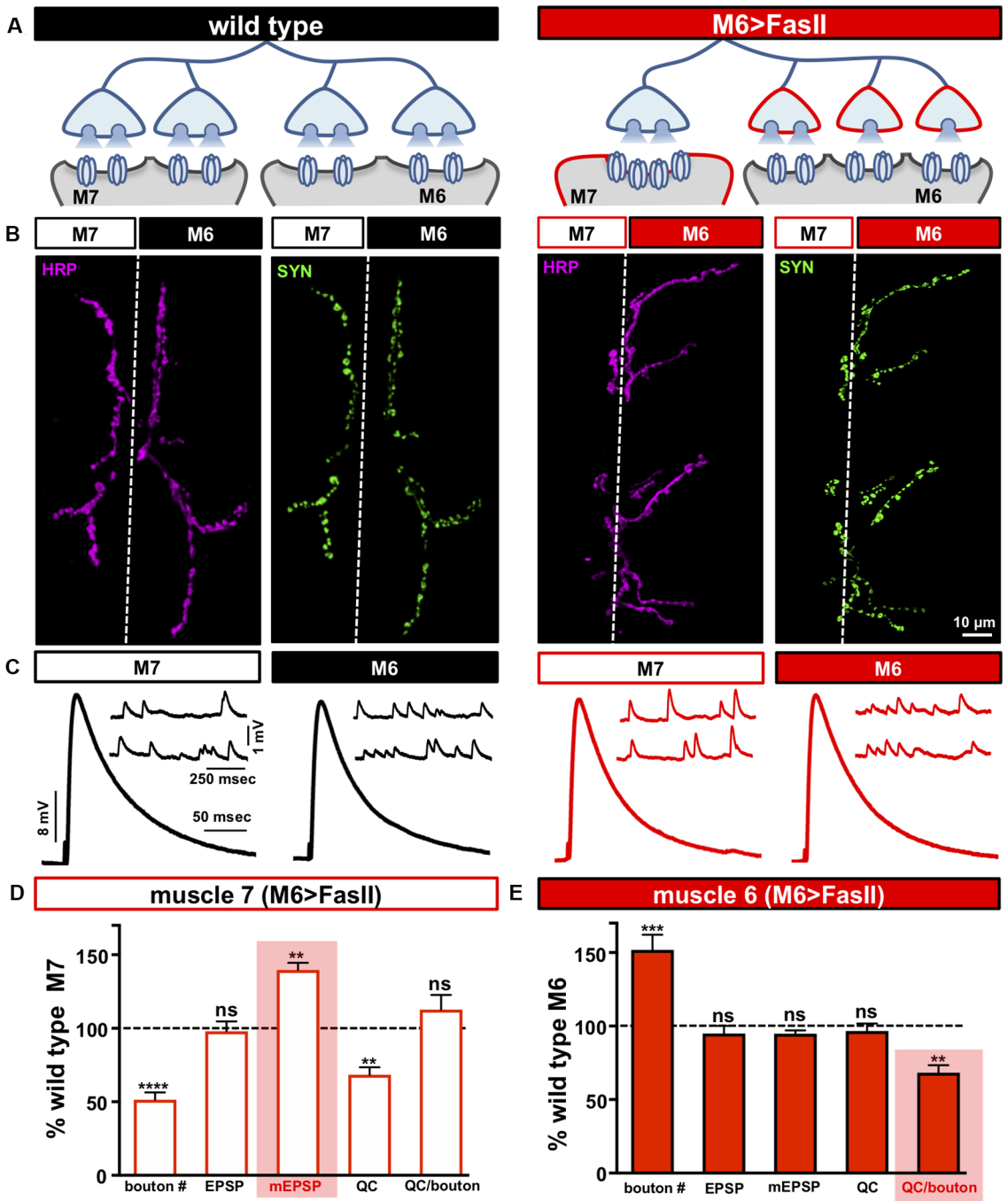

FIGURE 1 | Biased innervation at the neuromuscular junction (NMJ) elicits distinct target-specific homeostatic adaptations. **(A)** Schematic of a motor neuron innervating both muscle 6 and 7 at the *Drosophila* larval NMJ. Biased innervation is achieved by overexpressing the cell adhesion factor *FasII* specifically on muscle 6 using *H94-Gal4* (M6 >FasII: *w; UAS-FasII/+; H94-Gal4/+*). Red outlines highlight the likely synaptic compartment in which the adaptation occurs. **(B)** Representative images of muscle 6/7 neuromuscular junctions (NMJs) immunostained with antibodies that recognize the neuronal membrane (Horshradish Peroxidase; HRP) and synaptic vesicles (Synapsin; SYN) in wild type (w^{1118}) and M6 >FasII. Note that while boutons labeled by SYN puncta are roughly equally split between muscles 6 and 7 in wild type, M6 >FasII causes biased innervation on muscle 6 at the expense of muscle 7. **(C)** Representative electrophysiological traces of recordings from muscles 7 and 6 in wild type and M6 >FasII NMJs. Note that while EPSP amplitudes are similar across all muscles, miniature excitatory postsynaptic potentials (mEPSPs) are increased only on muscle 7 of M6 >FasII. **(D)** Quantification of bouton number, EPSP amplitude, mEPSP amplitude, quantal content, and quantal content normalized per bouton on muscle 7 in M6 >FasII. All values are normalized to the values at wild type muscle 7. Enhanced mEPSP amplitude (shaded bar) implies reduced quantal content and no change in quanta released per bouton. **(E)** Quantification of all values in **(D)** on muscle 6 of M6 >FasII normalized to wild type muscle 6 values. Note that the estimated quantal content per bouton (shaded bar) is significantly reduced. Error bars indicate ±SEM ($n \geq 16$; one-way ANOVA; **Supplementary Table S2**). $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$; ns, not significant.

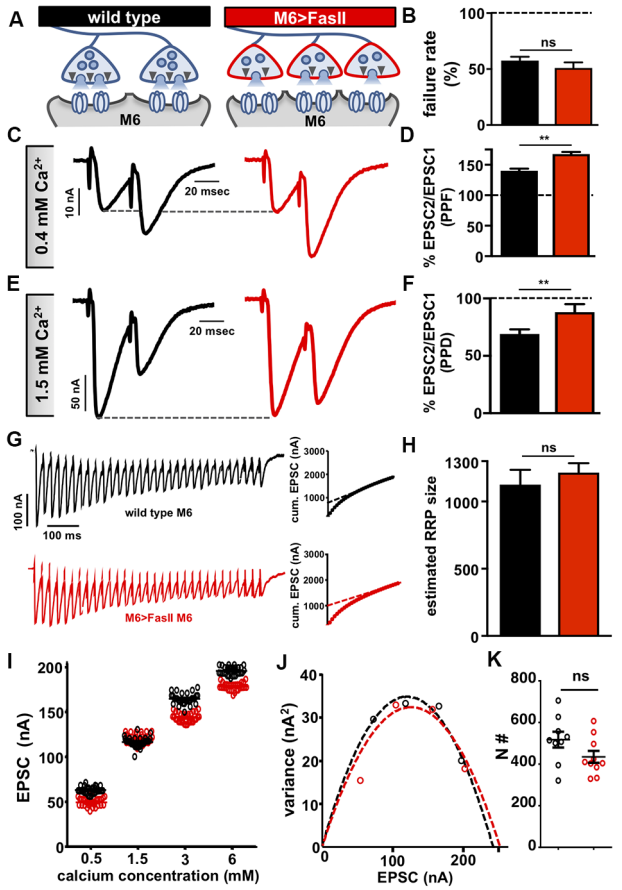

FIGURE 2 | Hyper-innervation elicits a homeostatic decrease in presynaptic release probability. **(A)** Schematic illustrating a reduction in readily releasable pool (RRP) size and functional release site number on hyper-innervated muscle 6. **(B)** Failure analysis reveals no significant change in failure rate on muscle 6 in M6 >FasII, consistent with unaltered quantal content on this target. **(C)** Representative paired-pulse EPSC traces at 0.4 mM extracellular Ca^{2+} with an interstimulus interval of 16.7 ms in the indicated genotypes. Increased paired-pulse facilitation (PPF) was observed on hyper-innervated targets, consistent with reduced release probability. **(D)** Quantification of PPF ratio (EPSC2/EPSC1). **(E)** Representative paired-pulse EPSC traces at 1.5 mM extracellular Ca^{2+} with an interstimulus interval of 16.7 ms in the indicated genotypes. Reduced paired-pulse depression (PPD) was observed on hyper-innervated targets, consistent with a reduced probability of release. **(F)** Quantification of PPD ratio (EPSC2/EPSC1) shows an increase. **(G)** Representative EPSC recordings of 30 stimuli at 3 mM extracellular Ca^{2+} during a 60 Hz stimulus train in the indicated genotypes. Insets represent the average cumulative EPSC plotted as a function of time. A-line fit to the 18^{th}–30^{th} stimuli was back-extrapolated to time 0. **(H)** The estimated size of the RRP is unchanged on muscle 6 in M6 >FasII compared with wild type, suggesting reduced RRP per bouton. **(I)** Scatter plot EPSC distribution of recordings on muscle 6 from wild type and M6 >FasII in the indicated extracellular Ca^{2+} concentrations. **(J)** Variance-mean plots for the indicated genotypes. Dashed lines are the best-fit parabolas to the data points. **(K)** The estimated number of functional release sites (N#) obtained from the variance-mean plots in **(J)** showing no significant difference between the genotypes. Error bars indicate ±SEM ($n \geq 9$; one-way ANOVA; **Supplementary Table S2**). $^{**}p < 0.01$; ns, not significant.

a 60× APO 1.4 NA oil immersion objective using separate channels with four laser lines (405, 488, 561, and 637 nm) at room temperature. Boutons were counted using NMJs stained with anti-Synapsin or -vGlut, co-stained with anti-HRP on muscle 6/7 of segment A2 and A3, considering each Synapsin or vGlut punctum to be a bouton. For fluorescence quantifications of postsynaptic GluRs and active zone proteins, all genotypes within a data set were immunostained in the same tube with identical reagents, then mounted and imaged in the same session. Z-stacks were obtained using identical settings for all genotypes with z-axis spacing between 0.15–0.2 µm within an experiment and optimized for detection without saturation of the signal. Maximum intensity projections were used for quantitative image analysis with the NIS Elements General Analysis toolkit.

To quantify the *sum punctum intensity*, the total fluorescence intensity signal of each punctum was calculated without regard to the area as described (Goel et al., 2019a). For each particular sample set, thresholds were optimized to capture the dynamic range of intensity levels within the wild type sample. This same threshold was then used to image all other genotypes in the sample set, and all intensities were normalized to wild type values within an experimental set. Active zones too closely spaced to be resolved (∼5% of all analyzed) were excluded from the analysis. Spot detection in the Nikon Elements Software was used to identify individual BRP and Cac puncta as it resolves closely spaced puncta more accurately compared to thresholding. Finally, to quantify *total intensity per NMJ*, the fluorescence intensity for each punctum (sum intensity) was added together across the entire NMJ. For calculation of BRP and Cac puncta density, the total number of puncta at a particular muscle was divided by the neuronal membrane area labeled by HRP spanning that muscle (Goel et al., 2019a). For image representation only, the gain and contrast were adjusted identically for all genotypes within a dataset. To show representative images of individual boutons, a particular area was selected from the entire NMJ (denoted with a white box) and rotated and cropped to demonstrate changes at boutons more clearly.

Electrophysiology

All dissections and recordings were performed in modified HL-3 saline (Stewart et al., 1994; Kikuma et al., 2017) containing (in mM): 70 NaCl, 5 KCl, 10 $MgCl_2$, 10 $NaHCO_3$, 115 Sucrose, 5 Trehalose, 5 HEPES, and 0.4 $CaCl_2$ (unless otherwise specified), pH 7.2. Neuromuscular junction sharp electrode (electrode resistance between 10–35 MΩ) recordings were performed on muscles 6 and 7 of abdominal segments A2 and A3 in wandering third-instar larvae (Kiragasi et al., 2017). Recordings were performed on an Olympus BX61 WI microscope using a 40×/0.80 NA water-dipping objective. Recordings were acquired using an Axoclamp 900A amplifier, Digidata 1440A acquisition system, and pClamp 10.5 software (Molecular Devices). Electrophysiological sweeps were digitized at 10 kHz and filtered at 1 kHz. Data were analyzed using Clampfit (Molecular devices), MiniAnalysis (Synaptosoft), and Excel (Microsoft) software.

Miniature excitatory postsynaptic potentials (mEPSPs) were recorded in the absence of any stimulation and cut motor axons were stimulated to elicit excitatory postsynaptic potentials

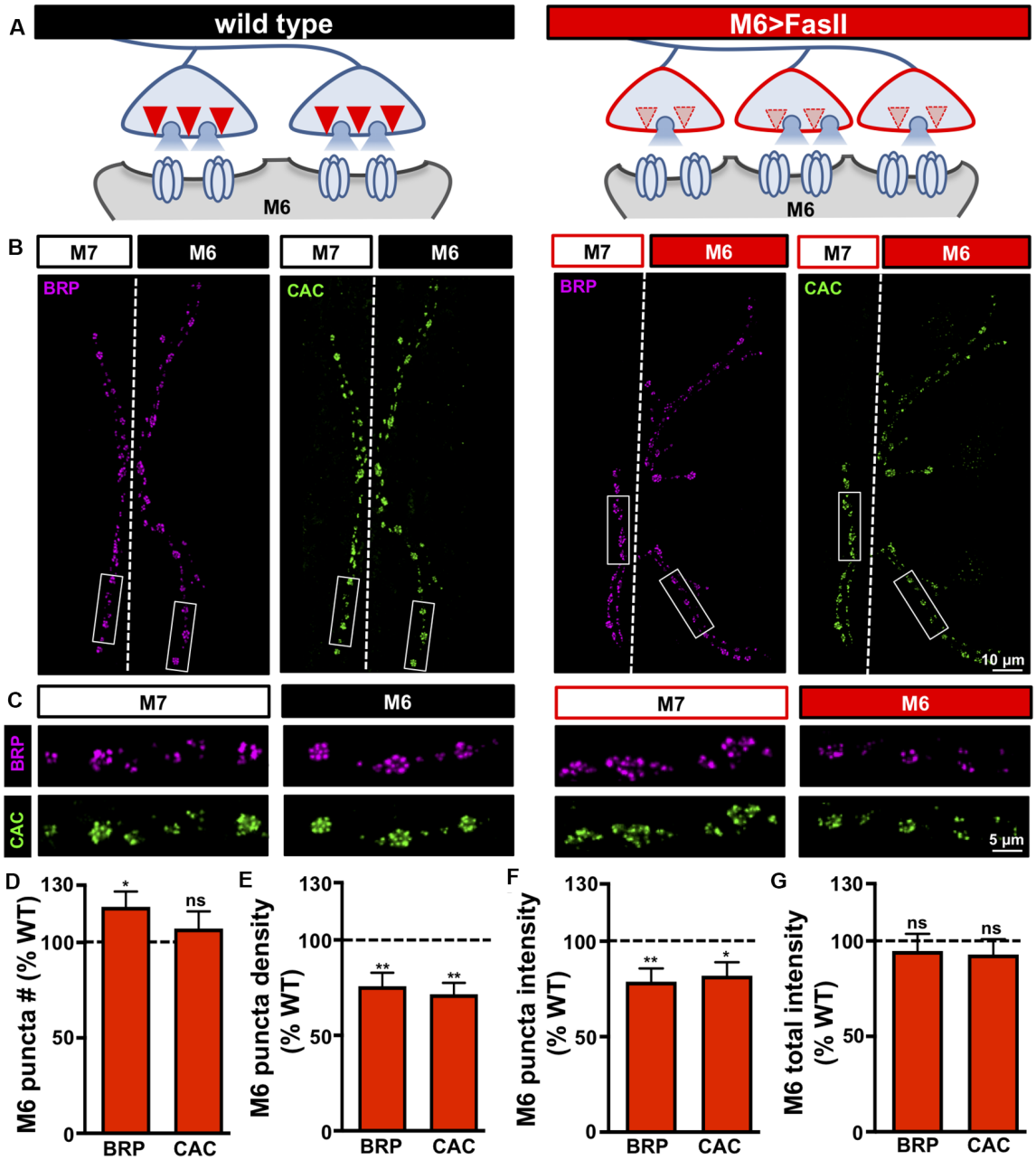

FIGURE 3 | Target-specific reductions in both active zone density and intensity at hyper-innervated NMJs. **(A)** Schematic illustrating a reduction in the number and intensity of active zones at individual boutons on hyper-innervated muscle 6. **(B)** Representative images of muscle 6/7 NMJs in the indicated genotypes (wild type: *cac*$^{sfGFP\text{-}N}$; M6 >FasII: *cac*$^{sfGFP\text{-}N}$; *UAS-FasII/+*; *H94-Gal4/+*) immunostained with antibodies against the active zone scaffold *bruchpilot* (BRP) and GFP to label endogenously tagged Ca^{2+} channels (CAC). **(C)** Individual boutons from selected areas (white rectangles) of NMJs stained with BRP and CAC in the indicated genotypes and muscles. Note the reduction in the number and intensity of BRP and CAC puncta specifically on muscle 6 in M6 >FasII, while no change is observed on muscle 7 relative to wild type controls. Quantification of BRP and CAC puncta number **(D)** and density **(E)** on muscle 6 in M6 >FasII normalized as a percentage of wild type muscle 6 values reveals a small but significant increase in BRP puncta number, while BRP and CAC puncta density is significantly reduced on muscle 6 in M6 >FasII. Quantification of BRP and CAC intensity **(F)** shows a significant reduction in muscle 6 in M6 >FasII, while the total fluorescence intensity of all BRP and CAC puncta summed across the entire muscle 6 NMJ **(G)** is unchanged compared to wild type muscle 6. Error bars indicate ±SEM ($n \geq 13$; one-way ANOVA; **Supplementary Table S2**). $^{*}p < 0.05$; $^{**}p < 0.01$; ns, not significant.

(EPSPs). An ISO-Flex stimulus isolator (A.M.P.I.) was used to modulate the amplitude of stimulatory currents. The intensity was adjusted for each cell, set to consistently elicit responses from both neurons innervating the muscle segment, but avoiding overstimulation. Average mEPSP, EPSP, and quantal content were calculated for each genotype. Muscle input resistance

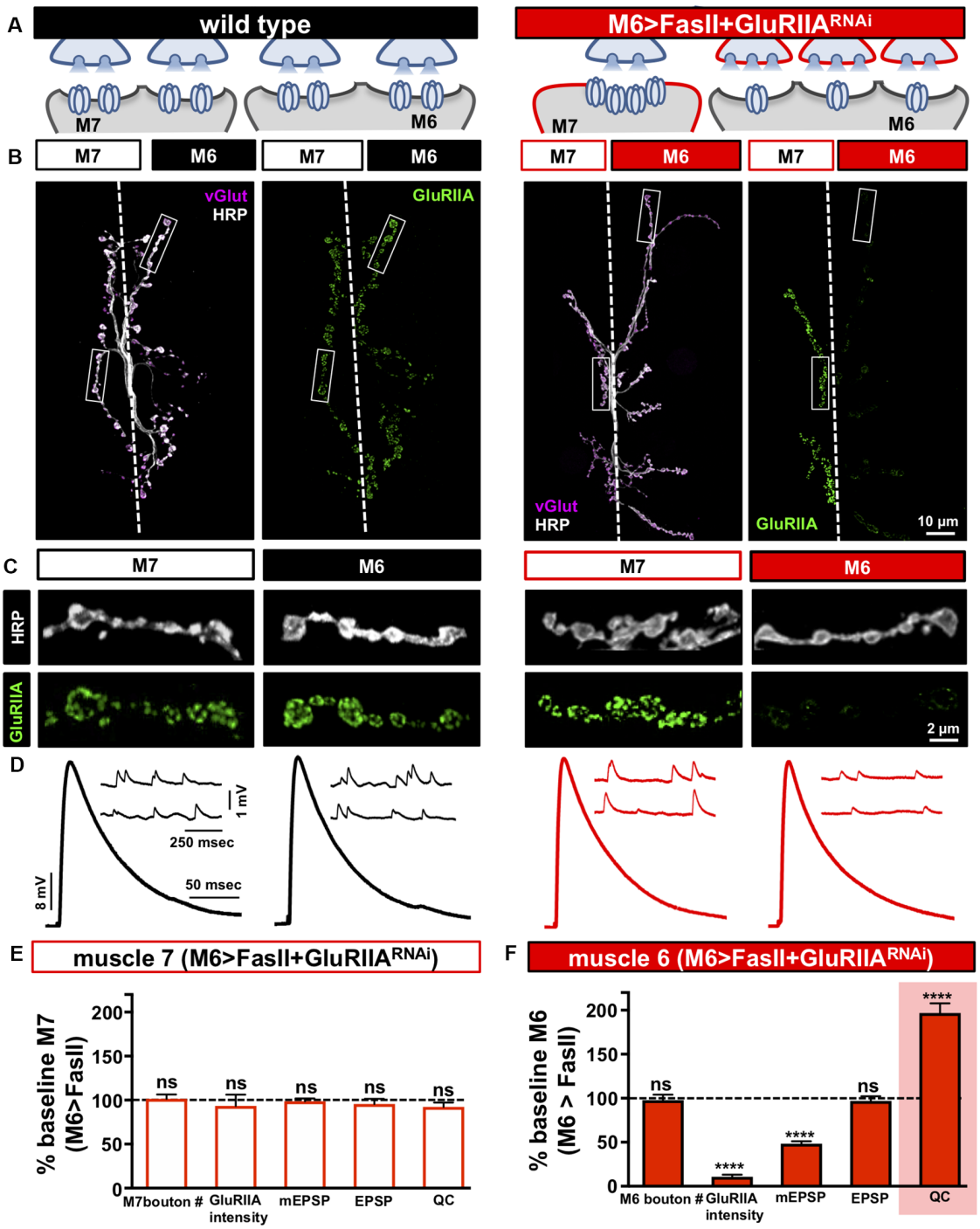

FIGURE 4 | Distinct target-specific adaptations balance hyper-innervation and GluR loss. **(A)** Schematic illustrating the dual manipulation used to both bias innervation and inhibit *GluRIIA* expression specifically on muscle 6 (M6 >FasII+GluRIIA^{RNAi}: *w;Tub-FRT-STOP-FRT-Gal4, UAS-FLP, UAS-CD8-GFP; H94-Gal4, nSyb-Gal80/UAS-FasII; UAS-GluRIIA*^{RNAi}). **(B)** Representative images of muscle 6/7 NMJs from the indicated genotypes immunostained with anti-HRP, -vGlut, and -GluRIIA. **(C)** Individual boutons from selected areas (white rectangles) of NMJs shown in (B). Note the enhanced GluRIIA levels on hypo-innervated muscle 7 with a loss on hyper-innervated muscle 6. **(D)** Electrophysiological traces of recordings from muscles 7 and 6 in the indicated genotypes. EPSP amplitudes on muscle 7 and 6 in M6 >FasII+GluRIIA^{RNAi} are similar to wild-type values. **(E)** Quantification of bouton numbers, GluRIIA puncta intensity, mEPSP amplitude, EPSP amplitude, and quantal content on muscle 7 in M6 >FasII+GluRIIA^{RNAi}. All values are normalized to baseline (M6 >FasII muscle 7); no significant differences are observed. **(F)** Quantification of all values in **(D)** on muscle 6 of M6 >FasII+GluRIIA^{RNAi} normalized to baseline (M6 >FasII muscle 6) values. Note that while GluRIIA levels and mEPSP amplitudes are significantly reduced, EPSP amplitude remains unchanged because of a homeostatic increase in quantal content, indicating presynaptic homeostatic potentiation (PHP) expression. Error bars indicate ±SEM ($n \geq 8$; Student's *t*-test; **Supplementary Table S2**). ****$p < 0.0001$; ns, not significant.

(R_{in}) and resting membrane potential (V_{rest}) were monitored during each experiment. Recordings were rejected if the V_{rest} was more depolarized than −60 mV, if the R_{in} was less than 5 MΩ, or if either measurement deviated by more than 10% during the experiment. Larvae were incubated with or without philanthotoxin-433 (PhTx; Sigma; 20 μM) and resuspended in HL-3 for 10 min as described (Frank et al., 2006; Dickman and Davis, 2009).

The readily releasable pool (RRP) size was estimated by analyzing cumulative EPSC amplitudes while recording in a two-electrode voltage clamp (TEVC) configuration as described (Goel et al., 2019c). Muscles were clamped at −80 mV and EPSCs were evoked with a 60 Hz, 60 stimulus train while recording in 3 mM Ca^{2+} HL-3. A-line fit to the linear phase (stimuli #18–30) of the cumulative EPSC data was back-extrapolated to time 0. The RRP value was estimated by determining the extrapolated EPSC value at time 0 and dividing this value by the average mEPSC amplitude.

Data used in the variance-mean plot was obtained from TEVC recordings using an initial 0.5 mM Ca^{2+} concentration, which was subsequently increased to 1.5, 3.0, and 6.0 mM through saline exchange using a peristaltic pump (Langer Instruments, BT100-2J). EPSC amplitudes were monitored during the exchange, and 30 EPSC (0.5 Hz stimulation rate) events were performed in each calcium condition (Li et al., 2018a). To obtain the variance-mean plot, the variance (squared standard deviation) and mean (averaged evoked amplitude) were calculated from the 30 EPSCs at each Ca^{2+} concentration. The variance was then plotted against the mean for each specific calcium condition using MATLAB software (MathWorks, USA). One additional data point, in which variance and mean are both theoretically at 0, was used for Ca^{2+}-free saline. Data from these five conditions were fit with a standard parabola (variance = $Q^*I_m - I_{m2}/N$), where Q is the quantal size, I_m is the mean evoked amplitude (x-axis), and N is the number of functional release sites. N, as a parameter of the standard parabola, was directly calculated for each cell by best parabolic fit.

Failure analysis was performed in an HL-3 solution containing 0.15 mM $CaCl_2$. At this extracellular Ca^{2+} concentration, approximately half of the stimulations evoked responses in the muscle in wild type larvae. A total of 40 trials (stimulations) were performed at each NMJ in all genotypes. The failure rate was obtained by dividing the total number of failures by the total number of trials (40). Paired-pulse recordings were performed at a Ca^{2+} concentration of 0.3 mM to assay facilitation (PPF) and 1.5 mM for depression (PPD). Following the first stimulation, a second EPSC was evoked at an interstimulus interval of 16.66 ms. Paired-pulse ratios were calculated as the difference between the second peak and the maximum value between both peaks (corresponding to the starting point of the second response) divided by the first amplitude.

Statistical Analysis

Data were analyzed using GraphPad Prism (version 7.0) or Microsoft Excel software (version 16.22). Sample values were tested for normality using the D'Agostino and Pearson omnibus normality test which determined that the assumption of normality of the sample distribution was not violated. Data were then compared using either a one-way ANOVA and tested for significance using a Tukey's multiple comparison test or using an unpaired 2-tailed Student's *t*-test with Welch's correction. All data are presented as mean ± SEM; *n* indicates sample number and *p* denotes the level of significance assessed as $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****); ns = not significant. Statistics of all experiments are summarized in **Supplementary Table S2**.

RESULTS

Target-Specific Mechanisms Maintain Stable Synaptic Strength at Hypo- and Hyper-innervated NMJs

We first sought to reproduce and confirm the biased innervation and synaptic electrophysiology reported in Davis and Goodman, 1998. At *Drosophila* larval NMJs, motor neurons distribute their synaptic terminals roughly evenly between two distinct targets—as demonstrated by the NMJs made onto muscles 6 and 7 (**Figure 1A**; left). This stereotyped pattern of innervation can be visualized by immunostaining the NMJ with antibodies that recognize the neuronal membrane (HRP) and synaptic vesicles (Synapsin; SYN), which demonstrates ∼60% boutons on the larger muscle 6 and ∼40% on the smaller muscle 7 (**Figure 1B**; left and **Supplementary Table S2**). To bias innervation on these targets, we used the *H94-Gal4* driver to drive expression of the cell adhesion molecule *Fasciculin II* (*FasII*) early in development selectively on muscle 6 (M6 >FasII; Davis and Goodman, 1998). Immunostaining of M6 >FasII NMJs confirmed biased innervation with ∼150% of boutons above controls on muscle 6 (hyper-innervated), and a parallel reduction of ∼50% in boutons on muscle 7 (hypo-innervated) (**Figures 1A–E**), consistent with the previous study (Davis and Goodman, 1998). However, despite these opposing changes in bouton numbers, electrophysiological recordings of M6 >FasII found that synaptic strength, measured by the excitatory postsynaptic potential (EPSP) amplitude, was similar on both targets and unchanged from their respective controls (**Figures 1C–E**). This implies target-specific mechanisms modulate neurotransmission on hypo- and hyper-innervated terminals to maintain stable NMJ strength.

To gain insight into how EPSP amplitudes remain similar to baseline values at NMJs with biased innervation, we next examined miniature neurotransmission. On hypo-innervated muscle 7, mEPSP amplitudes were significantly increased by ∼40% compared to baseline values (**Figures 1C,D**), as previously observed (Davis and Goodman, 1998). Quantal content (QC) was thus decreased by ∼40%, a value similar in magnitude to the reduction in bouton number (**Figure 1D**). In contrast, mEPSP amplitude was not significantly different on the hyper-innervated muscle 6 NMJ compared to baseline (**Figures 1C,E**), with no apparent change in quantal content (**Figure 1E**), as previously observed (Davis and Goodman, 1998). Finally, analysis of quantal content normalized per bouton on muscle 6 NMJs revealed an ∼30% reduction (**Figure 1E**),

suggesting a target-specific, homeostatic decrease in presynaptic neurotransmitter release, consistent with the results of single bouton recordings (Davis and Goodman, 1998). Together, this data indicates that distinct target-specific mechanisms operate to stabilize neurotransmission at hypo- vs. hyper-innervated NMJs.

A Homeostatic Increase in Postsynaptic GluR Abundance Stabilizes Synaptic Strength on Hypo-innervated Targets

It was previously reported that at hypo-innervated NMJs following M6 >FasII, levels of the postsynaptic GluR subunit GluRIIA were increased (Goel and Dickman, 2018). We, therefore, focused on postsynaptic adaptations to GluRs, as we considered two possible presynaptic changes unlikely. First, increased presynaptic vesicle size could in principle lead to enhanced glutamate emitted per vesicle, as has been documented in endocytosis mutants and following overexpression of the vesicular glutamate transporter (Daniels et al., 2004; Goel et al., 2019a). However, there is no evidence for endocytic defects or increased *vGlut* expression induced by the M6 >FasII manipulation. Second, although multivesicular release has been observed in some systems (Rudolph et al., 2015) and was raised as a possibility in the original study to potentially explain the increased quantal size (Davis and Goodman, 1998), multi-vesicular release at the fly NMJ is rarely if ever observed (Melom et al., 2013; Brusich et al., 2018). Hence, we focused on possible postsynaptic mechanisms to explain the increased mEPSP amplitude on hypo-innervated NMJs, which may parallel the ones that have been documented in mammalian forms of homeostatic receptor scaling (Turrigiano, 2008; Diering and Huganir, 2018). These include increases in the abundance, subtype, and/or functionality of additional postsynaptic GluRs, including GluRIIB-containing receptors, as enhanced levels of GluRIIA-containing GluRs were recently reported at hypo-innervated NMJs in *Drosophila* (Goel and Dickman, 2018; Goel et al., 2019b).

We, therefore, examined postsynaptic GluR levels in hypo-innervated targets induced by M6 >FasII. At the *Drosophila* NMJ, the postsynaptic response to glutamate is mediated by two subtypes of GluRs, GluRIIA- and GluRIIB-containing receptors. Both subtypes are composed of the essential subunits GluRIIC, GluRIID, and GluRIIE but differ in containing either GluRIIA or GluRIIB subunits (Qin et al., 2005; DiAntonio, 2006). We immunostained hypo-innervated NMJs using antibodies against GluRIIA, GluRIIB, and the common GluRIID subunits and observed an ∼45% decrease in the number of GluR puncta compared to wild type muscle 7 (**Supplementary Figures S1A,C**), reflecting reduced innervation. However, we found an increase in the intensity of all GluR subunits in hypo-innervated NMJs compared to wild type muscle 7 (**Supplementary Figures S1B,D**). In principle, the ∼55% increase in GluR abundance is sufficient to explain the increased quantal size and to offset the ∼40% reduction in quantal content to homeostatically maintain stable synaptic strength despite reduced innervation. Consistent with this, we observed no adaptations in the anatomical or functional number of release sites, nor in the size of the RRP (**Supplementary Figure S2**). These lines of evidence indicate that presynaptic terminals of hypo-innervated NMJs function similarly to wild type, with presynaptic neurotransmitter release onto the muscle 7 NMJ simply reduced by 40%. Thus, a ∼55% increase in postsynaptic GluR abundance per receptor field is sufficient to maintain synaptic strength at hypo-innervated NMJs without reason to invoke other homeostatic adaptations.

Hyper-innervation Induces a Homeostatic Decrease in Presynaptic Release Probability

We next sought to characterize the expression mechanism that enables stable neurotransmitter output on the hyper-innervated target. It was previously demonstrated that a homeostatic reduction in presynaptic release probability was expressed at hyper-innervated NMJs, where single bouton recordings measured a lower release probability for individual boutons (Davis and Goodman, 1998). Consistent with this conclusion, and in contrast to the adjacent hypo-innervated NMJs, we did not observe any significant changes in postsynaptic GluR levels (**Supplementary Figures S1E–H**). We next performed a series of electrophysiological assays to probe presynaptic function on the hyper-innervated NMJ. First, we used failure analysis to assess presynaptic release independently of miniature transmission by measuring the number of failed release events in very low extracellular Ca^{2+} concentrations (0.15 mM; see "Materials and Methods" section). We observed no significant difference in the failure rates on hyper-innervated NMJs compared to wild type (**Figure 2B**), consistent with overall quantal content being unchanged at these NMJs. Next, we probed short term plasticity by determining paired-pulse ratios in moderate and high extracellular Ca^{2+}. At 0.4 mM Ca^{2+}, we observed an increase in paired-pulse facilitation (PPF) at hyper-innervated NMJs compared to wild type (**Figures 2C,D**), while at 1.5 mM Ca^{2+}, paired-pulse depression (PPD) was reduced at hyper-innervated NMJs (**Figures 2E,F**). Since short term facilitation and depression vary inversely with release probability, enhanced PPF, and reduced PPD are indicative of reduced release probability (Regehr, 2012). While overall release probability, as calculated by failure analysis, is unchanged at hyper-innervated NMJs, the PPF/PPD findings may reflect altered short term Ca^{2+} and/or vesicle dynamics at individual release sites. Indeed, an inverse effect on short-term facilitation was reported in *rab3* mutants, which have reduced number but enhanced size of active zones (Graf et al., 2009). These results suggest that a target-specific, homeostatic decrease in presynaptic release probability at individual release sites serves to stabilize transmission at hyper-innervated NMJs.

Although the PPF/PPD recordings suggested reduced release probability at individual active zones of hyper-innervated terminals, the magnitude of the observed decrease (∼25%) was not sufficient to fully compensate for the ∼50% increase in bouton numbers. We found no change in the size of the RRP on hyper-innervated NMJs compared to wild type (**Figures 2G,H**), suggesting that the size of the RRP at individual boutons might

be reduced on muscle 6 of M6 >FasII NMJs. Finally, no change in the total number of functional release sites was observed on hyper-innervated targets (**Figures 2I–K**), indicating a reduction in the number of release sites participating in neurotransmission per bouton at hyper-innervated NMJs. Thus, a homeostatic adjustment in the release probability of individual active zones and the number of release sites per bouton selectively modulate transmission at hyper-innervated NMJs without measurably impacting release at adjacent hypo-innervated terminals.

A Target-Specific Reduction in Both Active Zone Density and Intensity Is Observed at Hyper-innervated NMJs

Our electrophysiological data above suggests a reduction in both release probability and the number of functional release sites at individual boutons of hyper-innervated NMJs. In principle, a target-specific reduction in the number and/or function of anatomical release sites could explain these electrophysiological properties. Also, recent evidence indicates that bi-directional changes in the size and nano-structure of active zone architecture at *Drosophila* NMJs can adjust release probability at individual active zones (Akbergenova et al., 2018; Böhme et al., 2019; Goel et al., 2019a; Gratz et al., 2019). We, therefore, characterized the number and intensity of individual active zones on hyper-innervated NMJs by immunostaining the central scaffold BRP and endogenously tagged CaV2.1 calcium channels (Cac^{sfGFP}; Gratz et al., 2019), defining each BRP punctum to be an active zone. Interestingly, while a ∼55% increase in bouton number was observed at hyper-innervated NMJs, the number of active zones was only increased by ∼20%, reflected in a concomitant decrease in active zone density (**Figures 3A–E**). Thus, hyper-innervated NMJs exhibit a target-specific reduction in the density of release sites that is sufficient in magnitude to limit the increase in active zones to only about 20% despite an ∼50% increase in innervation.

We also quantified the intensity of individual BRP puncta on hyper-innervated NMJs and observed an ∼20% decrease in the sum intensity of individual BRP puncta compared to wild type (**Figures 3B,C,F**). Similar results for puncta density and intensity were found for Cac^{sfGFP} (**Figures 3B–F**). Finally, given these reductions in the density and intensity of active zone components, the total intensity of both BRP and Cac^{sfGFP} per hyper-innervated NMJ was not significantly different from wild type despite the increase in their total number (**Figure 3G**). These results parallel recent studies that have shown that while the number and intensity of individual active zones can vary at NMJs, the total abundance of active zone protein remains constant (Graf et al., 2009; Goel et al., 2019a,b) or can reflect nanoscale remodeling of active zone components (Böhme et al., 2019; Mrestani et al., 2020). Together, hyper-innervated NMJs express a target-specific reduction in both the number and intensity of release sites per bouton and a parallel reduction in presynaptic release probability that stabilizes synaptic strength, while no reciprocal changes are observed at hypo-innervated counterparts.

Distinct Target-Specific Adaptations Can Homeostatically Balance Hyper-innervation and GluR Perturbation

When biased innervation of the NMJ is induced through M6>FasII, the hypo-innervated target responds by homeostatically increasing GluR abundance, while the subset of motor neuron terminals that hyper-innervate the adjacent target selectively reduce the number and apparent abundance of active zone components. In our final set of experiments, we sought to determine whether the target-specific homeostatic adaptations triggered by biased innervation could be balanced with an additional target-specific homeostatic challenge. PHP is a well-studied form of homeostatic plasticity at the *Drosophila* NMJ. Here, rapid pharmacological or chronic genetic manipulations that diminish postsynaptic GluR functionality trigger a trans-synaptic retrograde signaling system that homeostatically increases presynaptic glutamate release to maintain stable synaptic strength (Frank et al., 2020). Recently, it was demonstrated that GluR knockdown specifically on muscle 6 can trigger PHP selectively at the subset of synapses innervating muscle 6 without influencing transmission at the synaptic terminals of the same motor neuron that innervate the adjacent muscle 7 (Li et al., 2018a), demonstrating a remarkable degree of compartmentalized expression of PHP. We combined these manipulations to induce a simultaneous challenge of biased innervation and GluR loss using *FasII* overexpression combined with *GluRIIA* knockdown selectively on muscle 6 (referred to as M6 >FasII+$GluRIIA^{RNAi}$; see "Materials and Methods" section for details). We first tested whether the combined manipulation was successful by assaying synaptic growth and GluRIIA levels. Indeed, we observed the expected increase and decrease in bouton numbers on muscles 6 and 7 respectively, with a near absence of GluRIIA immunostaining selectively on muscle 6 (**Figures 4B–F**). Thus, target-specific, homeostatic challenges of biased innervation and GluR loss can be simultaneously induced by overexpressing *FasII* and $GluRIIA^{RNAi}$ selectively on muscle 6.

We next performed synaptic electrophysiology at both targets. On the hypo-innervated muscle 7 of M6 >FasII+$GluRIIA^{RNAi}$, neurotransmission was indistinguishable from M6 >FasII alone, with elevated mEPSP amplitudes, stable EPSP amplitudes, and reduced quantal content observed (**Figures 4D,E**). In contrast, on the hyper-innervated muscle 6 of M6 >FasII+$GluRIIA^{RNAi}$, mEPSP amplitudes were selectively reduced due to GluR knockdown, but synaptic strength was maintained at baseline levels due to a homeostatic increase in quantal content (**Figures 4D,F**). This demonstrates that PHP can be robustly expressed and balanced with the adaptations necessary to adjust release for hyper-innervation in a target-specific manner, without any apparent changes in transmission at adjacent synapses of the hypo-innervated muscle 7. Finally, we tested whether PHP can be acutely induced and balanced at hypo-innervated NMJs after the adjustments made at muscle 6 of M6>FasII+$GluRIIA^{RNAi}$. We applied sub-blocking concentrations of the GluR venom philanthotoxin-433 (PhTx) at

NMJs for 10 mins. This acutely induced PHP at wild type NMJs, with reduced mEPSP amplitude but EPSP amplitudes unchanged from baseline due to a rapid, homeostatic increase in quantal content (**Supplementary Figures S3A–D**). Application of PhTx to M6 >FasII+GluRIIA^{RNAi} NMJs had no significant change in mEPSP amplitude or quantal content at muscle 6 due to GluRIIA knockdown (**Supplementary Figures S3A,B,D**). However, PhTx application also induced robust PHP at muscle 7 NMJs in M6 >FasII+GluRIIA^{RNAi}, with a significant reduction in mEPSP amplitude but normal EPSP amplitude due to enhanced quantal content (**Supplementary Figures S3A,C,E**). These results demonstrate that presynaptic release sites at terminals of the same neuron can be selectively modulated with exquisite target specificity to compensate for GluR loss and can be superimposed with the homeostatic plasticity induced by biased innervation.

DISCUSSION

Recent studies have shed light on how neurotransmission is stabilized when synaptic growth and function is challenged (Davis and Müller, 2015; Li et al., 2018b; Goel et al., 2019a,b; Frank et al., 2020). However, less is known about how this stability is maintained when neuronal terminals confront diverse and even opposing challenges in synaptic growth and function. Here, we have utilized a manipulation pioneered by Davis and Goodman (1998) to induce biased innervation and provoke target-specific plasticity and combined this with acute and chronic challenges to postsynaptic GluR function at distinct targets shared by individual neurons. These experiments have revealed two distinct target-specific mechanisms that enable stable transmission despite biased innervation, operating at either pre- or postsynaptic compartments, and that can be balanced with postsynaptic GluR perturbation. Importantly, these processes occur independently, without impacting transmission within the same neuron on neighboring synapses made on the adjacent target. This demonstrates a remarkable degree of compartmentalized autonomy in homeostatic signaling and suggests the independence of local and global homeostats that work in concert to balance synaptic strength.

Target-Specific Homeostatic Scaling of Postsynaptic GluR Receptors

We took advantage of a previously established manipulation to bias synaptic innervation using the target-specific expression of the trans-synaptic cell adhesion protein FasII (Davis and Goodman, 1998). On the hypo-innervated target, a selective upregulation in postsynaptic GluR abundance was elicited sufficient in magnitude to offset reduced neurotransmitter release and stabilize synaptic strength. This scaling of GluR abundance parallels a well-established mechanism of homeostatic synaptic plasticity in mammalian neurons termed *homeostatic receptor scaling* (Turrigiano, 2008; Chowdhury and Hell, 2018; Diering and Huganir, 2018). Although optogenetic activity can be used to provoke GluRs to rapidly traffic at the fly NMJ in ways that parallel the dynamics of GluRs in mammalian dendritic spines (Ljaschenko et al., 2013), the GluR scaling revealed in this study is unique. GluRs at the *Drosophila* NMJ are typically quite stable, and this receptor stasis may reflect a fundamental property of NMJs, where postsynaptic receptors have half-lives of ∼7 days in rodents (Salpeter and Harris, 1983) and over 24 h in flies (Rasse et al., 2005). While NMJ receptors appear to be relatively stable under basal conditions and even in mutants in which synaptic transmission and growth are perturbed (Saitoe et al., 2001; Lee et al., 2013; Goel et al., 2019b), there is emerging evidence that specific challenges, including activity, injury, and disease, can provoke relatively rapid remodeling of neurotransmitter receptors at postsynaptic compartments of the NMJ (Rich and Lichtman, 1989; Palma et al., 2011; Ljaschenko et al., 2013; Perry et al., 2017; Goel and Dickman, 2018). The temporal regulation and dynamics of the hypo-innervation-induced GluR plasticity are unclear but likely to be intertwined with NMJ development and growth.

The induction mechanisms that enable reduced innervation to be sensed and to ultimately instruct an adaptive increase in postsynaptic GluR abundance are unclear. Two different types of motor neurons innervate most muscles in *Drosophila*, called type Is (phasic) and type Ib (tonic; Atwood et al., 1993; Kurdyak et al., 1994; Lnenicka and Keshishian, 2000). Differences in GluR composition have been noted at terminals of Is and Ib inputs (Schmid et al., 2008), and there is evidence that these motor neuron subtypes may possess different plasticity rules (Newman et al., 2017; Li et al., 2018b; Aponte-Santiago et al., 2020). Although no major differences in the adaptations related to hypo- and hyper-innervation were observed between Is and Ib inputs (Davis and Goodman, 1998), future work may uncover input-specific distinctions. It is notable that while hypo-innervation in the M6>FasII manipulation elicits GluR scaling, a variety of mutations that lead to synaptic undergrowth do not consistently change receptor levels (Kaufmann et al., 2002; Marqués et al., 2002; Banovic et al., 2010; Goel et al., 2019b). Further, mutations that severely reduce neurotransmitter release, including *synaptotagmin* and *complexin* mutants, do not change GluR levels (Saitoe et al., 2001; Huntwork and Littleton, 2007; Lee et al., 2013). Hence, hypo-innervation and/or reduced neurotransmitter release alone is unlikely to be sufficient to induce postsynaptic GluR scaling. Rather, this form of homeostatic plasticity may be dependent on the phenomenon of biased innervation between two targets shared by a single neuron itself, implying some signaling between the motor neuron and/or the adjacent muscles is involved. What is clear is that the postsynaptic signal transduction system that mediates hypo-innervation-dependent GluR scaling is distinct from that which mediates retrograde PHP signaling, as GluR scaling can still be expressed in conditions in which postsynaptic PHP signaling is blocked (Goel and Dickman, 2018). Finally, it is interesting to note that the induction of PHP signaling is initiated by loss or blockade of GluRs, while the ultimate expression mechanism of GluR scaling involves a homeostatic upregulation in the abundance of these same GluRs at postsynaptic compartments. Thus, postsynaptic GluRs are central targets for both the induction and expression of homeostatic synaptic plasticity.

Target-Specific Modulation of Active Zones

In contrast to the exclusively postsynaptic adaptation observed in response to reduced innervation, an entirely presynaptic mechanism stabilizes synaptic strength at hyper-innervated muscles, expressed by a target-specific reduction in the number and intensity of active zone components. Although a similar reduction in the abundance of active zone proteins at individual release sites has recently been found in mutations that cause synaptic overgrowth at the NMJ (Goel et al., 2019a,b), the adaptations observed in the case of hyper-innervation are distinct in that they are: (1) target-specific; and (2) involve a reduction in active zone density in addition to their apparent intensity. Although increased fluorescence intensity is typically interpreted to reflect enhanced protein abundance, a recent study using Localization Microscopy showed that increased active zone intensity may instead reflect a more compact nanoscopic arrangement (Mrestani et al., 2020). Nonetheless, it is remarkable that both the number and intensity of active zone components can be selectively reduced and calibrated at hyper-innervated terminals without any apparent changes at adjacent terminals shared by the same neuron on the hypo-innervated target. This suggests the intriguing possibility that target-specific modulation of active zone structure might homeostatically control a cargo delivery process at synapses. One attractive candidate pathway may involve the lysosomal adaptor Arl-8. Arl-8 regulates the delivery of synaptic vesicle and active zone cargo to synapses (Klassen et al., 2010; Vukoja et al., 2018), and was recently shown to promote the delivery of synaptic cargo necessary to remodel active zones during PHP (Goel et al., 2019a). Because active zone components are remodeled during PHP (Weyhersmüller et al., 2011; Böhme et al., 2016; Goel et al., 2017; Gratz et al., 2019) through an *arl-8* dependent mechanism (Goel et al., 2019a), and PHP can be expressed at a subset of terminals with target-specificity (Li et al., 2018a), it is tempting to speculate that Arl-8 may also be involved in the target-specific reduction in active zones following hyper-innervation.

Biased Innervation, Presynaptic Homeostatic Plasticity, and Information Transfer at Synapses

Global synaptic strength is established during development through intrinsic genetic programs and a dialogue between pre- and post-synaptic compartments. Robustness in this process is ensured by signaling systems that can sense and adapt to deviations outside of physiological ranges, such as reductions or enhancements in synaptic growth (Tripodi et al., 2008; Yuan et al., 2011; Keck et al., 2013; Goel et al., 2019b,c). Superimposed on this foundation are forms of plasticity such as PHP, which appear to operate as independent homeostats to maintain stable information transfer at synapses and within neural circuits. Presynaptic terminals of a neuron, therefore, do not function as unitary computational units but are rather compartmentally specialized and flexible according to the physiologic needs of their targets during development and following homeostatic challenges. In addition to this target-specificity, there is also evidence for input-specificity across dendrites that can homeostatically modulate strength in rodent hippocampal neurons (Katz et al., 2009; Jia et al., 2010; Stuart and Spruston, 2015; Letellier et al., 2019). This remarkable control of synaptic activity enables the flexibility to locally adjust synaptic strength through input- and target-specificity while stabilizing overall network activity and information processing.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

PG and DD designed the research. PG, SN, KC, CC, and XL performed the research. PG analyzed the data. PG and DD wrote the article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fncel.2020.00196/full#supplementary-material

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REVIEW
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Synaptic Plasticity in Cortical Inhibitory Neurons: What Mechanisms May Help to Balance Synaptic Weight Changes?

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Inhibitory neurons play a fundamental role in the normal operation of neuronal networks. Diverse types of inhibitory neurons serve vital functions in cortical networks, such as balancing excitation and taming excessive activity, organizing neuronal activity in spatial and temporal patterns, and shaping response selectivity. Serving these, and a multitude of other functions effectively requires fine-tuning of inhibition, mediated by synaptic plasticity. Plasticity of inhibitory systems can be mediated by changes at inhibitory synapses and/or by changes at excitatory synapses at inhibitory neurons. In this review, we consider that latter locus: plasticity at excitatory synapses to inhibitory neurons. Despite the fact that plasticity of excitatory synaptic transmission to interneurons has been studied in much less detail than in pyramids and other excitatory cells, an abundance of forms and mechanisms of plasticity have been observed in interneurons. Specific requirements and rules for induction, while exhibiting a broad diversity, could correlate with distinct sources of excitatory inputs and distinct types of inhibitory neurons. One common requirement for the induction of plasticity is the rise of intracellular calcium, which could be mediated by a variety of ligand-gated, voltage-dependent, and intrinsic mechanisms. The majority of the investigated forms of plasticity can be classified as Hebbian-type associative plasticity. Hebbian-type learning rules mediate adaptive changes of synaptic transmission. However, these rules also introduce intrinsic positive feedback on synaptic weight changes, making plastic synapses and learning networks prone to runaway dynamics. Because real inhibitory neurons do not express runaway dynamics, additional plasticity mechanisms that counteract imbalances introduced by Hebbian-type rules must exist. We argue that weight-dependent heterosynaptic plasticity has a number of characteristics that make it an ideal candidate mechanism to achieve homeostatic regulation of synaptic weight changes at excitatory synapses to inhibitory neurons.

Keywords: inhibitory neurons, neocortex, hippocampus, synaptic plasticity, homeostasis, homosynaptic plasticity, heterosynaptic plasticity

INTRODUCTION

Inhibition in cortical networks serves a multitude of functions, including balancing and restricting the spread of excitation (Wehr and Zador, 2003; Okun and Lampl, 2008; Ozeki et al., 2009; Moore et al., 2018), organizing neuronal activity in temporal and spatial patterns (Klausberger and Somogyi, 2008; Adesnik and Scanziani, 2010; Cardin, 2018; Unal et al., 2018), and shaping response selectivity of cortical neurons (Vidyasagar et al., 1996; Monier et al., 2003; Barnes et al., 2015). Adaptive fine-tuning of inhibition, necessary for achieving these functions, is mediated by synaptic plasticity. Plasticity of inhibitory systems can be mediated by changes at inhibitory synapses and also by changes at excitatory synapses at inhibitory neurons. Here, we consider that latter locus: plasticity at excitatory synapses to inhibitory neurons.

Plasticity of excitatory synaptic transmission to interneurons has been investigated in much less detail than in pyramidal neurons and other excitatory neurons. Inhibitory interneurons, while representing about 10–20% of the total number of neurons in different cortical areas, express a remarkable diversity of types, serving distinct roles in the operation of cortical networks and characterized by a distinct morphology, electrophysiology, and pattern of protein expression (Kawaguchi and Kubota, 1997; Markram et al., 2004; Ascoli et al., 2008; Battaglia et al., 2013; Druckmann et al., 2013; Jiang et al., 2015; Tremblay et al., 2016). Research has shown that excitatory inputs to these diverse types of inhibitory neurons express a multitude of forms and mechanisms of plasticity (reviewed in Bischofberger and Jonas, 2002; Galván et al., 2011; Kullmann and Lamsa, 2011; Laezza and Dingledine, 2011; Topolnik, 2012; Pelkey et al., 2017; Topolnik and Camiré, 2019), including Hebbian-type plasticity (Alle et al., 2001; Lamsa et al., 2005; Lu et al., 2007; Le Roux et al., 2013). Hebbian-type rules introduce positive feedback on synaptic weight changes: Potentiation of a synapse makes it more effective in evoking action potentials and, thus, increases the probability of further potentiation of that synapse. Similarly, depression of a synapse decreases its chances to evoke a spike and be potentiated, thus increasing the probability of its further depression. This positive feedback makes synaptic weights prone to runaway potentiation or depression and eventual saturation, which may impair the ability of synapses for further adaptive changes and compromise stability of operation of neurons and neuronal networks. However, synaptic weights in real inhibitory neurons do not express runaway dynamics and remain within an operational range, and neuronal networks of the brain operate in a regime of balanced excitation and inhibition. This implies the existence of additional plasticity mechanisms that counteract the tendency for runaway dynamics of synaptic weights introduced by the positive feedback of Hebbian-type rules. We argue that such homeostatic regulation of synaptic weight changes can be achieved by heterosynaptic plasticity at excitatory synapses to inhibitory neurons.

To appreciate the context in which candidate homeostatic mechanisms operate, we first consider diverse forms and mechanisms of plasticity of excitatory synapses at various types of inhibitory neurons. The diversity of plasticity forms in interneurons highlights the need for a generic and robust homeostatic mechanism(s). A candidate mechanism that fulfills these requirements is calcium-dependent heterosynaptic plasticity. Therefore, we next consider calcium sources that can trigger plasticity in interneurons and evidence for heterosynaptic plasticity, including a novel form of weight-dependent heterosynaptic plasticity that we have recently described for major electrophysiological types of inhibitory neurons. Finally, we discuss how these diverse forms of plasticity might affect the overall excitatory drive of inhibitory neurons, and which of these forms of plasticity could contribute to homeostatic regulation of synaptic weights of excitatory inputs to inhibitory neurons.

DIVERSE FORMS AND MECHANISMS OF PLASTICITY OF EXCITATORY INPUTS TO INHIBITORY NEURONS

Research into plasticity of excitatory synaptic transmission to inhibitory neurons has revealed that mechanisms of plasticity can be connection-specific, i.e., determined by the identity of both the presynaptic and postsynaptic cells. Therefore, the description of plasticity studies below is organized both historically and by specific connections, defined by the location of interneurons and the source of axons forming the synapses. Throughout the description, we accentuate two further points that are important for the purposes of this review. First, that outcome of plasticity experiments is typically not uniform, implying that, in addition to the type of connection and detail of the plasticity induction protocol, further factors are involved in determining whether the result will be long-term potentiation (LTP), long-term depression (LTD), or no change. Second is the issue of input specificity of plastic changes. Because heterosynaptic plasticity might play a central role in balancing synaptic changes, we point to evidence for heterosynaptic changes even when considering results of studies aimed at investigation of homosynaptic plasticity (see **Box 1** for definitions and discussion).

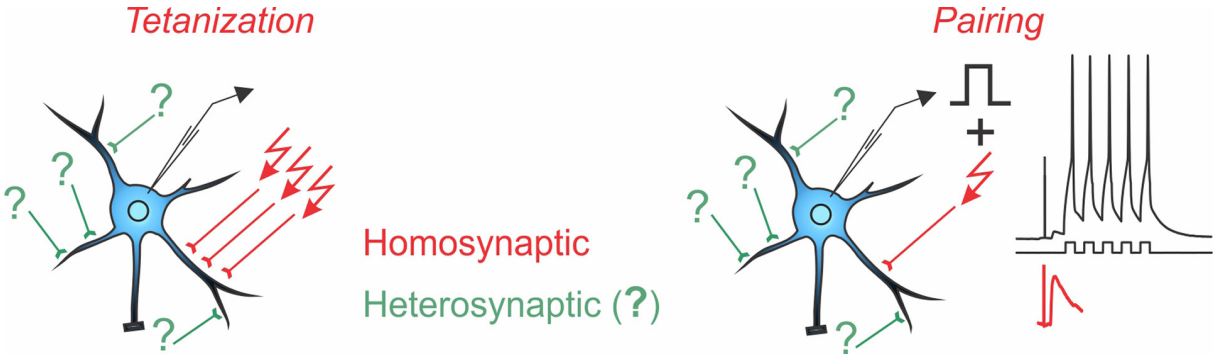
The hippocampus represents a classical experimental system to study synaptic plasticity, and it has been a structure of choice for most studies of plasticity of excitatory synaptic transmission to inhibitory interneurons (**Figure 1** and **Table 1**).

Plasticity of Excitatory Inputs to CA1 Interneurons in the Hippocampus

Diverse Forms of Calcium-Dependent Plasticity in CA1 Interneurons

The first intracellular study of plasticity of excitatory transmission to inhibitory neurons in the hippocampus aimed to reveal whether changes in excitability of interneurons could contribute to regular tetanus-induced LTP of field potentials (Taube and Schwartzkroin, 1987). Afferent tetanization did not change excitability of basket cells recorded at the border of str. pyramidale and oriens of the CA1; however, it induced plasticity of subthreshold EPSPs.

Induction of plasticity in fast-spiking (FS) neurons from CA1 str. pyramidale required $[Ca^{2+}]_i$ rises. Plastic changes induced by either high-frequency tetanization combined with



BOX 1 | Homosynaptic and Heterosynaptic Plasticity.
Two main protocols are most commonly used for the induction of long-term plasticity of synaptic transmission: afferent tetanization and pairing. Either protocol can induce *homosynaptic plasticity*, changes at synapses that were activated during the induction (inputs in red with arrows denoting stimulation during the induction). Co-occurring alongside homosynaptic plasticity is *heterosynaptic plasticity*, defined as changes at inputs that were not stimulated during the induction protocol (inputs in green, marked with question marks).
A complicating factor in the concept of homosynaptic plasticity is the nature of "input-specificity." Conventionally, plasticity is called input-specific if no changes are observed in an independent test input, not stimulated during the induction, i.e., no heterosynaptic changes. In a strict sense, input specificity means changes *only* at activated synapses and not at any other of the hundreds or thousands of synapses on the postsynaptic neuron, only a few of which were contributing to the tested heterosynaptic response. Assessing changes at all unstimulated inputs is technically intractable. At the same time, all studies that specifically addressed changes at nearby synapses found that input-specificity breaks down at short distances (Schuman and Madison, 1994; Engert and Bonhoeffer, 1997; Royer and Paré, 2003). Also at synapses distant from those stimulated during the induction, heterosynaptic plasticity is often induced, e.g., by calcium rises produced by back-propagating spikes (for further discussion see Chistiakova et al., 2014, 2015). Note that unlike input specificity, the wording "homosynaptic plasticity" makes no assumptions and has no implications about possible changes (or absence of changes) at other synapses that were not tested. Therefore, in this review, we use "homosynaptic plasticity" to refer to changes at synapses activated during the induction, and "heterosynaptic plasticity" to refer to changes at synapses that were not activated during the induction.

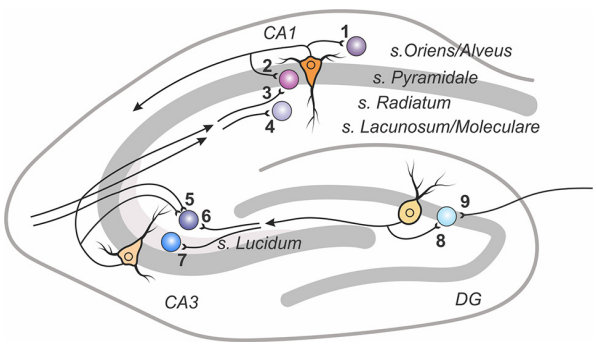


FIGURE 1 | A scheme of excitatory connections to inhibitory interneurons in the hippocampus, in which synaptic plasticity was studied. Excitatory contacts onto interneurons of specified lamina in the dentate gyrus, areas CA1 and CA3 are displayed with reference number corresponding to cited research in **Table 1**.

depolarization of the recorded interneuron (Cowan et al., 1998) or pairing low-frequency stimulation with depolarization (Wang and Kelly, 2001) were prevented by adding BAPTA to the intracellular pipette solution. Blockade of NMDARs with APV did not prevent the pairing-induced LTP but reduced its magnitude (Wang and Kelly, 2001). This suggests that, although NMDARs contribute to calcium entry in FS str. pyramidale interneurons, their involvement is not critical, and rises of $[Ca^{2+}]_i$ necessary for triggering LTP could be achieved by engaging sources other than NMDARs. In CA1 str. oriens interneurons, induction of LTP by theta-burst stimulation paired with postsynaptic depolarization was not affected at all by NMDAR blockade but prevented by blockers of group I/II metabotropic glutamate receptors (mGluR) or selective mGluR1a antagonists (Perez et al., 2001). These neurons express calcium-permeable AMPA receptors (CP-AMPARs), which might have contributed to the Ca^{2+} influx needed for triggering LTP. Notably, the ability of the above theta-burst protocol to induce plasticity was "connection-specific"—it was effective in the str. oriens interneurons but did not induce plasticity in str. radiatum interneurons (Perez et al., 2001).

A characteristic morphological feature of inhibitory neurons is aspiny or sparsely spiny dendrites. Because, in excitatory neurons, spines are considered as the morphological substrate for restricting the spread of synaptically induced $[Ca^{2+}]_i$ rises, thus restricting plasticity to the activated synapses, it was proposed that, in aspiny dendrites, input-specificity of plastic changes might be compromised. Indeed, direct tests revealed a lack of input specificity of synaptic changes in CA1 inhibitory neurons (McMahon and Kauer, 1997; Cowan et al., 1998). In basket and bi-stratified neurons from str. radiatum, high-frequency tetanization induced predominantly LTD, which was not restricted to the tetanized input but could spread to nonstimulated synapses (McMahon and Kauer, 1997). In FS neurons from str. pyramidale, high-frequency tetanization combined with depolarization could induce LTP, LTD, or lead to no changes in both tetanized and nontetanized pathways in all possible combinations (Cowan et al., 1998).

TABLE 1 | Plasticity in inhibitory neurons in the hippocampus and neocortex.

Reference Cortical region (connection #)	Layer cell type	Stim site/Input	Induction protocol	Blocker/Agonist in bath	Homosynaptic LTP, LTD, No (out of *N* cases)	Heterosynaptic	Mechanisms/Involved receptors/Ca++ source/Cascades	Pre/Post (measure)	Species Age rec T°
Taube and Schwartzkroin (1987) *Hippocampus CA1* (**3**)	Pyramidale/ oriens border basket	S. radiatum	HFS: 100 stimuli @100 Hz	—	3 LTP; 3 LTD; 6 No (*n* = 12)	—	—	—	Guinea pig adult 35°C
McMahon and Kauer (1997) *Hippocampus CA1* (**4**)	Radiatum basket, bistratified	S. radiatum Schaffer collat	HFS: 100 stimuli @100 Hz, x2@0.05 Hz; Pairing: 60 stim @1 Hz +depolariz. to −10 mV	PTX	HFS: 3 LTP; 32 LTD; 14 No (*n* = 49); Pairing did not induce plasticity	Heterosynaptic LTD (8 out of 8 tested)	—	—	SD rat P16-26 29–31°C
Cowan et al. (1998) *Hippocampus CA1* (**3**)	Pyramidale FS	S. radiatum	HFS: 40 stimuli @100 Hz, x4@0.1 Hz, alone or with depolarization	Bic or PTX	HFS: 0 LTP; 6 LTD; 5 No; (*n* = 11); HFS + Dep: 10 LTP; 17 LTD; 8 No (*n* = 35)	HFS: 2 LTP; 3 LTD; 6 No; (*n* = 11); HFS + Dep: 9 LTP; 18 LTD; 8 No (*n* = 35)	Ca++ dependent, blocked by BAPTA	—	Wistar rat P17–25 29–31°C
Wang and Kelly (2001) *Hippocampus CA1* (**3**)	Pyramidale FS non-pyramidal neurons	Schaffer collaterals/ comissural fibers	Pairing: 30 stimuli at 1 Hz with depolarization to 0 mV	—	LTP (to about 200%);	—	Ca++ dependent, blocked by BAPTA; reduced (150% ctrl instead) by APV; CaMKII-dependent, blocked by Ca-binding peptide or autoinhibitory CaMKII(281–301) in the pipette, potentiation by activation of CaMKII occludes LTP;	—	SD rat P18–22 31°C
Perez et al. (2001) *Hippocampus CA1* (**1, 4**)	Oriens or radiatum	S. oriens or radiatum; minimal stim	TBS: 4 stimuli @100 Hz paired with 60 ms steps to −20 mV; x5@5 Hz; x3@0.033 Hz;	—	Oriens: LTP (*n* = 15); No changes if TBS alone (*n* = 8) or depolarization steps alone (*n* = 8); radiatum: No changes (*n* = 8)	—	NMDA-independent; prevented by mGluR-I/II blockers or selective mGluR1a antagonist;	S. oriens: pre (failure rate);	Rat P18–21 22–24°C

(Continued)

TABLE 1 | Continued

Reference Cortical region (connection #)	Layer cell type	Stim site/Input	Induction protocol	Blocker/Agonist in bath	Homosynaptic LTP, LTD, No (out of *N* cases)	Heterosynaptic	Mechanisms/Involved Receptors/Ca++ source/Cascades	Pre/Post (measure)	Species Age rec T°
Lamsa et al. (2005) *Hippocampus CA1* (**4**)	Radiatum	S. radiatum	Pairing: 120 stimuli at 2 Hz at *V*h = 0 mV, pulses (a) or continuous (b)	PTX + CGP–52432	Pairing (a) 16 LTP, 0 LTD, 14 No (n = 30); Pairing (b) 17 LTP, 0 LTD, 28 No (n = 45)	Excluded from analyses ("LTP defined as >25% pathway-specific potentiation")	Ca++ dependent; NMDA-dependent	Post (no PPR changes)	SD rat P21–28
Lamsa et al. (2007) *Hippocampus CA1* (**1, 2**)	Pyramidale oriens//alveus bi-stratified, axo-axonic, basket; RS or FS	Alveus	HFB: 5 stim@100 Hz x5@4-5 Hz, x4@0.1 Hz; Pairing: 100 stimuli at de- or hyperpolariz. Phase of a 4 Hz sine wave; HFS: 100 stimuli @100 Hz x2@0.1 Hz	PTX + CGP–52432	HFB, single stimuli or HFS, paired with depolarization (current injection or strong stimuli): No changes; with hyperpolarization: LTP ("anti-Hebbian")	Not considered; though clear cases for heterosynaptic LTP in scatters	Ca++ dependent; NMDA independent; CP-AMPA dependent;	—	SD rat P21–28 31–32°C
Topolnik et al. (2006) *Hippocampus CA1* (**1**)	Oriens//alveus	S. oriens	TBS: 4 stimuli @100 Hz paired with 60 ms steps to −20 mV; x5@5 Hz; x3@0.033 Hz;	—	LTP (n = 5); LTP if ERK, Srk or intracellular Ca++ release alone blocked; but LTD if combinations are blocked, or TRP receptor blocked	—	Ca++ imaging; mGluR1α and mGluR5 involved in fast and slower Ca++ signals; sources of intracellular Ca++ increase; LTP induction by TBS with dep pulses: still LTP if ERK, Srk or intracellular Ca++ release alone blocked; but LTD if combinations are blocked; also, block of TRK receptors -> LTD	Pre (failure rate)	SD rat P15–23 31–33°C
Jia et al. (2010) *Hippocampus CA1* (**1**)	Oriens, nicotine-sensitive cells; PV−; some are NPY+,CR+, SST+,VIP+	S. oriens	HFS: 100 stimuli @100 Hz, VC −70 mV	APV; Bic; MLA; atropine	No (n = 4) in 'control cocktail'; LTP in 10 μM (n = 4) or 1 μM (n = 5) nicotine;	—	NMDA-independent; required nicotine receptors (with the used blockers); Ca++ dependent, blocked by BAPTA, but not by ryanodine or nifedipine; nicotine induces Ca++ influx *via* activation of non α-7 AChRs, also with APs blocked;	—	SD rat P18–54; 30°C

(Continued)

TABLE 1 | Continued

Reference Cortical region (connection #)	Layer cell type	Stim site/Input	Induction protocol	Blocker/Agonist in bath	Homosynaptic LTP, LTD, No (out of *N* cases)	Heterosynaptic	Mechanisms/Involved receptors/Ca++ source/Cascades	Pre/Post (measure)	Species Age rec T°
Nissen et al. (2010) *Hippocampus CA1* (**1, 2, 3, 4**)	Pyramidale, radiatum, oriens; PV+; NPY+; SST+; CBR1+; axo-axonic, basket, bi-stratified, non-basket	S. oriens/ alveous; in some expts ctrl eld in S. radiatum	HFS: 100 stim @100 Hz x2@0.05 Hz at −70 or −90 mV; TBS: 5 stimuli @100 Hz, x4@5 Hz, x5@0.05 Hz	APV; PTX + CGP–55845	Perisomatic-targeting (*n* = 14): LTP in PV+ CB1R− (7/8); No in PV− CB1R+ (6/6); Dendrite-targeting, bistratified PV+: 0 LTP, 5 LTD, 2 NO (*n* = 7); PV− CB1R+: No (5/5); CB1R+: No plasticity (14/14) after HFS or TBS even without APV	Excluded from analyses (LTP defined as >25% pathway-specific potentiation)	CP-AMPAR involved; CP-AMPARs present in PV+ (low RI, *n* = 45 inputs) but not in CB1R+ cells (high RI > 0.5 in 25/30 inputs)	—	SD rat P21–28; 31–33°C
Szabo et al. (2012) *Hippocampus CA1* (**1, 4**)	Radiatum (ivy cells; Schaffer-Collaterals associated cells) oriens (O-LM cells)	S. radiatum (for ivy cells); S. oriens (for O-LM cells)	TBS: 5 stimuli @100 Hz, x4@4 Hz, x10@0.05 Hz; sometimes with depolarization pulses	APV; PTX + CGP–55845; AM-251	LTP in Ivy NOS+ cells (6/6) and O-LM SM+ cells (6/6); No LTP if TBS paired with depolarization (Ivy 5/5; O-LM 7/7); No LTP in SCA CCK+ CB1R+ cells (*n* = 5 TBS; *n* = 5 TBS with depolarization)	Not considered	CP-AMPARs are necessary; present in ivy and O-LM cells	Pre (CV^{-2})	SD rat P21–28; 31–33°C
Griguoli et al. (2013) *Hippocampus CA1* (**1**)	Oriens SST+ cells	S. oriens/alveus	HFS: 100 stimuli @100 Hz x2@0.1 Hz + hyperpolarization to −90 −100 mV	APV; gabazine + CGP–54656	LTP in control (*n* = 17); with α7-nAChRs blocked: 6 LTP, 11 No (*n* = 17); with α7-nAChRs, mGluR-I and mGluR1/5 blocked: No changes (*n* = 15); α7−/− mice: 1 LTP, 17 No (*n* = 18)	—	Ca++ influx through α7 nicotinic CP-AChRs is necessary for 'anti-Hebbian' LTP	pre	Mouse P14–21; C57BL/6 or alpha7−/−

(Continued)

TABLE 1 | Continued

Reference Cortical region (connection #)	Layer cell type	Stim site/Input	Induction protocol	Blocker/Agonist in bath	Homosynaptic LTP, LTD, No (out of *N* cases)	Heterosynaptic	Mechanisms/Involved receptors/Ca++ source/Cascades	Pre/Post (measure)	Species Age rec T°
Le Roux et al. (2013) *Hippocampus CA1* (**2, 3**)	Pyramidale PV+	S. radiatum for FF Schaffer collateral inputs; S. oriens/alveus for FB inputs	400 stimuli @5 Hz, at 0 mV for Hebbian; at −90 mV for anti-Hebbian; Also: 900 pulses @ 0.1, 1, 5, 20 Hz; and 100 stim@100 Hz x5;	Bic	Anti-Hebbian: LTP in both FF and FB inputs (n = 11; n = 9); Hebbian: LTP in FB only (n = 8), but No in FF (n = 13);	No, input-specific only	Anti-Hebbian required CP-AMPA; Hebbian required NMDA; differential frequency-dependence (BCM-curve)	Post (PPR, NASPM-block; responses to uncaged Glu)	Mouse P17–23; 31°C
Camiré and Topolnik (2014) *Hippocampus CA1* (**1**)	Oriens FS, basket and bi-stratified	S. oriens/ alveus, distal inputs	TBS: 3 stimuli @100 Hz, x8@4 Hz, x3@0.033 Hz	Gabazine + CGP–55845	Sub-threshold TBS, small amplitude Ca++ transients: LTP (n = 7); Supra-threshold TBS, large supralinear Ca++ signals: LTD (n = 7); if supralinear Ca++ summation is blocked with CPA: LTP after strong TBS	—	Ca++ signals (imaging): CP-AMPARs; less NMDA; small contribution of L-type VGCC; supralinearity of Ca++ signals produced by burst stimulation was eliminated by NASPM block of CP-AMPARs; CPA or ryanodine block of Ca-induced Ca++ release; but not by blocking NMDARs or VGCC (L,T,R)	—	Mouse P13–21; 30–33°C
Nicholson and Kullmann (2014) *Hippocampus CA1* (**1**)	Oriens regular firing	Alveus/oriens border	HFS: 100 stimuli @100 Hz x2@0.05 Hz; APs only: 500 pA 500 ms depolarization x20@0.2 Hz	APV; PTX + CGP–55845	HFS: LTP (n = 29); APs only: LTP (n = 15);	No, input-specific after HFS (yes, after AP only)	Ca++ dependent (blocked by 25 mM BAPTA); no involvement of NO; no involvement of TRPV1; occlusion between HFS-induced and AP-only induced LTP	HFS: pre (failure rate, PPR, spont freq) APs only: pre (PPR, spont freq)	Mouse P21–25
Nicholson and Kullmann (2017) *Hippocampus CA1* (**1**)	Oriens regular firing	Alveus/oriens border	HFS: 100 stimuli @100 Hz x2@0.05 Hz; APs only: 500 pA 500 ms depolarization x20@0.4 Hz	APV; PTX + CGP–55845	HFS and APs-only induced LTP	No, input-specific after HFS (yes, after AP only)	T-type Ca++ channels contribute to both HFS-induced and APs-only induced LTP	—	Mouse P16–23
Maccaferri et al. (1998) *Hippocampus CA3* (**7**)	Lucidum or border to radiatum	DG mossy fibers	Tetanic stimulation of MF, parameters not specified	Bic; APV	0 LTP; 6 LTD; 3 No (n = 9)	—	NMDA-independent; Ca++ independent, NOT occluded by forskolin	Presyn (failure rate)	SD rat; P14–20; 24°C

(Continued)

TABLE 1 | Continued

Reference Cortical region (connection #)	Layer cell type	Stim site/Input	Induction protocol	Blocker/Agonist in bath	Homosynaptic LTP, LTD, No (out of *N* cases)	Heterosynaptic	Mechanisms/Involved receptors/Ca++ source/Cascades	Pre/Post (measure)	Species Age rec T°
Laezza et al. (1999) *Hippocampus CA3* (**5**)	Radiatum	CA3 pyramid. layer; continuum of CP-AMPAR — CI-AMPAR synapses	HFS: 30 stimili @100 Hz, x3@0.1 Hz	Bic; APV	CP-AMPAR synapses: LTD (12/12); CI-AMPAR synapses: 7 LTP; 3 No (n = 10)	—	LTD at CP-AMPAR synapses: Ca++ dependent, abolished by 30 mM BAPTA or clamp at +40 mV; mGluR7-dependent, prevented by group II/III mGluR antagonist LY341495 (without affecting basal transmission)	Presyn (failure rate)	SD rat, P10–16
[-1pt] Laezza and Dingledine (2004) *Hippocampus CA3* (**5**)	Radiatum	CA3 pyramidal layer; continuum of CP-AMPAR — CI-AMPAR synapses; No correlation with NMDA-component	HFS: 30 stimili @100 Hz, x3@0.1 Hz; Pairing: 120 stim @1 Hz, at −25 mV;	Bic	CP-AMPARs: LTP after HFS at −30 mV (n = 6) or Pairing at −25 mV (n = 4); HFS at 0 mV 1 LTP; 4 LTD; 1 No (n = 6); HFS at −70 mV 1 LTP; 4 LTD; 2 No (n = 7); LTD after HFS at −30 mV with 30 mM BAPTA (n = 4); CI-AMPARs: No changes after HFS at −30 mV (n = 6);	—	CP-AMPAR with NMDAR synapses: both LTP and LTD were NMDAR-dependent; but with intracellular BAPTA HFS induced LTD; CP-AMPAR synapses lacking NMDAR: LTD was induced by pairing	—	SD rat P9–12; RT
[-1pt] Lei and McBain (2002) *Hippocampus CA3* (**7**)	Lucidum	DG mossy fibers; continuum of synapses: CP-AMPAR with low NMDA/AMPA — CI-AMPAR with high NMDA/AMPA	HFS: 100 stimili @100 Hz, x3@0.1 Hz	Bic; Glycine	LTD at both CI and CP-AMPAR synapses (n = 5; n = 6); with APV: No changes at CI-AMPAR synapses (n = 6); LTD at CP-AMPAR synapses (n = 7)	—	Ca++ dependent (blocked with 20 μM BAPTA); NMDAR-dependent in CI-AMPAR synapses; NMDAR-independent in CP-AMPAR synapses	—	SD rat; P16–20
[-1pt] Lei and McBain (2004) *Hippocampus CA3* (**7**)	Lucidum	DG mossy fibers	HFS: 100 stimili @100 Hz, x3@0.1 Hz	Bic	LTD at both CI- and CP-AMPAR synapses	—	CI-AMPAR syn: NMDA-dependent; AMPA-trafficking; CP-AMPAR syn: NMDA-independent;	CI-AMPAR syn: post; CP-AMPAR syn: pre; (CV, PPR, NMDA-resp, use-depend AMPAR-block)	SD rat; P16–20; 22–24°C

(Continued)

TABLE 1 | Continued

Reference Cortical region (connection #)	Layer cell type	Stim site/Input	Induction protocol	Blocker/Agonist in bath	Homosynaptic LTP, LTD, No (out of *N* cases)	Heterosynaptic	Mechanisms/Involved receptors/Ca++ source/Cascades	Pre/Post (measure)	Species Age rec T°
Pelkey et al. (2005) *Hippocampus CA3* (**7**)	Lucidum	DG mossy fibers	HFS: 100 Hz 1 s, x3@0.1 Hz	Bic; APV	LTD in control (n = 10); after reduction by mGluR7 agonist AP4 responses recover to control after HFS (is it "LTP" ?)	No; no changes at synapses from CA3 collaterals	LTD blocked by mGluR7-antagonist MCOG; PKC-dependent. APV all times in the bath -> only NMDA-independent component	Presyn (PPR, CV, failure rate)	Mouse C57BL/6 P12–22; 22–25°C, some at 33–35°C
Galván et al. (2008) *Hippocampus CA3* (**6**)	Lacunosum/ moleculare	DG mossy fibers; 18/28 CI-AMPARs; 10/28 CP-AMPARs	HFS: 100 stimuli @100 Hz **+ depolarization**, x3@0.1 Hz	Bic; APV	With CP-AMPARs blocked by PhTx: synapses with mostly CI-AMPARs showed associative LTP (n = 11, inp-specific, no in C-A inputs); synapses with initially stronger but blocked CP-AMPAR component showed No changes (5/7) or LTD (2/7) of the remaining CI-AMPAR mediated component; Without Ph-Tx: 25 LTP; 2 LTD; 5 No (n = 32)	No; no changes at comiss/ associate synapses from CA3 in experiments with PhTx	Ca++ dependent. Prevented by hyperpolarization (n = 10), L-type VGCC (n = 9); by 20 mM BAPTA in 11/15 cells. NMDA-R independent; requires mGluR1, IP3 and RyR; with mGluR1, IP3 blocked or RyR-release depleted LTD was induced.	Both LTP and LTD: pre; (PPR, CV, failure rate)	SD rat, P22+4; 33 ±1 °C;
Galván et al. (2015) *Hippocampus CA3* (**6**)	Radiatum or lacunosum/ moleculare	DG mossy fibers	HFS: 100 stimuli @100 Hz + **depolarization**, x3@0.1 Hz	—	in Bic + APV: LTP (n = 6)	No; no changes in RC inputs in experiments with blockers of CaMKII or PKC	LTP in MF: NMDA-independent; not blocked by CaMKII inhibitors KN62 or KN-93; requires postsynaptic PKC: LTD with intracellular PKC blocker chelerythrine (n = 9);		SD rat, P35+5; 33 ± 1°C

(Continued)

TABLE 1 | Continued

Reference Cortical region (connection #)	Layer cell type	Stim site/Input	Induction protocol	Blocker/Agonist in bath	Homosynaptic LTP, LTD, No (out of *N* cases)	Heterosynaptic	Mechanisms/Involved receptors/Ca++ source/Cascades	Pre/Post (measure)	Species Age rec T°
Galván et al. (2015) *Hippocampus CA3* (**5**)	Radiatum or lacunosum/ moleculare	CA3 pyramid. layer, recurr collaterals; 19/26 CI– 5/26 CP– AMPAR syn; (2:atypical IV)	HFS: 100 stimuli @100 Hz + **depolarization**, x3@0.1 Hz	—	in Bic + APV: CP-AMPARs: LTD (5/5); CI-AMPARs: transient potentiation (10/19), No (9/19); Bic (no APV): LTP at CI-AMPAR (*n* = 8);	No; no changes in MF inputs in experiments with blockers of CaMKII or intracellular PKC	LTP in RC CI-AMPARs was blocked by: hyperpolarization to −100 mV; APV; intracellular 20 mM BAPTA; bath application of CaMKII inhib KN-62 or KN-93; intracellular PKC blocker chelerythrine; not blocked: by mGluR1 or mGluR5 blockers	Presyn (PPR, CV)	SD rat, P35+5; 33±1°C
Pan et al. (2019) *Hippocampus CA3* (**7**)	Lucidum	DG mossy fibers; only CP-AMPARs included, with Rectification Index <0.3	HFS (not specified)	PTX; APV	WT mice (controls, *n* = 84): 0 LTP, 70 LTD, 14 No; TrkB −/− or blocked: 5 LTP, 4 LTD, 8 No (*n* = 17); TrkB/PLC signaling blocked: 6 LTP, 8 LTD, 11 No (*n* = 25); BDNF −/− or scavenged: 3 LTD, 10 No (*n* = 13); CB1R antagonists: 4 LTP, 0 LTD, 14 No (*n* = 18); CB1R−/−: 3LTP, 2LTD, 1No	—	LTD at CP-AMPARs: NMDA-independent; prevented or converted to a mix LTP/LTD/No, if BDNF/TrkB/PLC signaling is blocked or impaired; or CB1Rs are blocked or deleted.	Presyn (PPR)	Mouse WT or conditnl TrkB−/− BDNF−/− CB1R−/− P21–29; Room T°
Alle et al. (2001) *Hippocampus Dentate gyrus* (**8**)	DG basket cells	DG mossy fibers; connected pairs granule-basket or extracell stim	HFS: 25stim@30 Hz, x12@0.33 Hz, x3@0.011; associative HFS: + BS spikes by depolariz. Pulses; nonassociative HFS: BS at −70 mV	—	LTP after associative aHFS; LTD after non-associative nHFS	—	LTP attenuated by 30 mM BAPTA (not by 10 mM) and reduced by by PKC-antagonist bisindolylmaleimide; not by PKA blocker H-89	Both LTP and LTD: presyn, (failure rate, CV, PPR)	Wistar rat P18–25; 34 ± 2°C

(Continued)

TABLE 1 | Continued

Reference Cortical region (connection #)	Layer cell type	Stim site/Input	Induction protocol	Blocker/Agonist in bath	Homosynaptic LTP, LTD, No (out of *N* cases)	Heterosynaptic	Mechanisms/Involved receptors/Ca++ source/Cascades	Pre/Post (measure)	Species Age rec T°
Sambandan et al. (2010) *Hippocampus Dentate gyrus* (**8, 9**)	FS perisomatic inhibitory neurons (PII)	DG mossy fibers (MF): CP-AMPARs and NMDAR; perforant path from enthorhinal ctx (PP): CI-AMPARs and low levels of NMDARs	BFS: 25 stim@30 Hz, x12@0.33 Hz, x3@0.033 Hz; associative BFS: PP (*dt* = 10 ms) + MF, induced AP bursts; "nonassociative": PP or MF + APs induced with 0–2 ms delay by depolariz. (*note: slower kinetics of PP may require different timing*)	Bic or SR95531	Associative BFS: LTP in MF, No changes in PP with 10 ms delay PP-MF; No LTP with <5 ms or >15 ms delay (*n* = 11); LTP at MF after pairing MF + depolarization-induced spikes at about 0 ms lag; No LTP at PP	No	LTP at MF synapses: NMDA-independent, requires CP-AMPA; depends on spike timing, few-ms window for induction by MF+PP pairing	—	Wistar rat P17–24; 30–34°C
[-1pt] Hainmüller et al. (2014) *Hippocampus Dentate gyrus* (**8**)	FS basket perisomatic inhibitory neurons (PII)	DG mossy fibers; connected pairs granule-basket or extracell stim	BFS: 25 stim@30 Hz, x12@0.33 Hz; associative BFS: + BS spikes by depolariz. Pulses with 1–3 ms delay; nonassociative BFS: same BFS but PII held at VC −70 mV	—	LTP after associative aBFS; LTD after nonassociative nBFS; LTP and LTD were independent, can be induced one after the other	—	Ca++ dependent; blocked by BAPTA but not by EGTA; Major Ca++ source during bursts is CP-AMPARs then NMDARs, while mGluRs, VDCCs or Ca++ stores contribute less; notably, Ca-response to single APs was not much affected by any of these; mGluR1/5 supported LTP but prevented LTD (*via* PKC activation); switch enabling MF-LTP	—	Wistar rat P17–23; 30–34°C
[-1pt] Lu et al. (2007) *somatosensory cortex*	L2/3 FS, LTS;	L2/3 pyramids; NMDAR-component about 3× stronger in PC-LTS than in PC-FS synapses	STDP 5 pre + post APs at 20 Hz, x12@0.2 Hz; pre-post delays: ±8 and ±25 ms; tested up to ±100 ms	—	LTS: LTP at +8 ms (*n* = 21); LTD at −8 ms (*n* = 12); No at +25, −25 ms (*n* = 5, 5); FS: LTD at +8 ms or −8 ms (*n* = 22, 19); No at +25 or −25 ms (*n* = 6, 8)	—	LTS: both LTP and LTD NMDAR-dependent; not sensitive to mGluR blockade by MCPG; FS: LTD did not require NMDAR; but prevented by MCPG	LTS: presyn; FS: postsyn; (CV , PPR)	SD rat, P13–16; 32–34°C
[-1pt] Chen et al. (2009) *somatosensory cortex*	L2-L4 non-FS SST+ PV−	L2-L4; 5–10 mV EPSPs; NMDA + AMPA components	TBS: 5 stimuli @100 Hz, x20@5 Hz, x6-10 times @0.1 Hz	PTX	LTP (*n* = 9); only STP, no LTP after 2–3 TBS episodes (*n* = 9); no LTP after HFS 100 Hz 1s x3 (*n* = 6);	—	NMDA-independent (*n* = 12); Ca++ independent, not blocked by 30 mM BAPTA + nimodipine; nor by *Vh* = −90 mV during TBS; blocked by incubation in PKA-inhibitors	Presyn (PPR)	Mouse P15–45, median P21 Room T°

(Continued)

TABLE 1 | Continued

Reference Cortical region (connection #)	Layer cell type	Stim site/Input	Induction protocol	Blocker/Agonist in bath	Homosynaptic LTP, LTD, No (out of *N* cases)	Heterosynaptic	Mechanisms/Involved receptors/Ca++ source/Cascades	Pre/Post (measure)	Species Age rec T°
Sarihi et al. (2008) *visual cortex*	L2/3 FS mostly PV+ basket; nonFS bitufted or bipolar	L4, half-maximal EPSPs	TBS: 4 stimuli @100 Hz, x10@5 Hz, x3@0.1 Hz + depolariz. to 0 mV ;	—	FS: 14 LTP, 0 LTD, 5No (n = 19); less LTP after TBS at −70 mV (6/17 cells); no LTP after depolarization alone (0/8 cells); non-FS: 6 LTP, 0 LTD, 11 No (n = 17);	—	FS: LTP is Ca++ dependent (blocked by 10 mM BAPTA, n = 8); but did not depend on NMDAR (APV, n = 8) or L,T type VGCC (nimodipine, Ni++, mibefradil; n = 11, 9, 9); required mGluR5 but not mGluR1; required PLC-IP3 system and release from internal Ca++ stores; after eye opening (P12–15) LTP did not depend on age	FS: postsyn (PPR, CV)	Mouse P12–43, most P16–19 29–31°C
Lefort et al. (2013) *visual cortex, (monocular V1)*	L4 FS	Connected pairs star pyramids -> FS (mostly FS->SP; occasional reciprocal SP->FS)	HFS: 10 spikes @50 Hz, x20@0.1 Hz in FS + subthreshold depolarization with 1–2 occasional spikes in SP; *note* that FS is postsyn, so mostly postsyn (FS) spiking	—	P16–17 no net changes in FS (119 ± 7.22%, n = 6); P22–23 net potentiation in FS (185 ± 45%, n = 7), reduced but not completely blocked by GABAB blocker CGP52432 (about 145%, from figure, n = 6);	*Heterosynaptic induction*? changes after postsynaptic FS firing, with only occasional spikes in presynaptic SP	Reduced but not completely blocked by GABAB blocker CGP52432;	Postsyn (CV)	Rat P15–23 35°C
Chistiakova et al. (2019) *visual cortex*	L1-5 FS; non-FS; diverse morpho-logical types;	Two bipolar electrodes near recording site	Pairing: synaptic stimuli to one input followed (10 ms) by 5 APs @100 Hz, x10@1 Hz, x3@0.017 Hz; Intracellular tetanization (IT): 5 APs @100 Hz, x10@1 Hz, x3@0.017 Hz, without synaptic stimuli	—	Pairing: 5 LTP, 2 LTD, 3 No (n = 10; net LTP); LTP in both FS and non-FS cells	Pairing, un-paired inputs: 3 LTP, 1 LTD, 6 No (n = 10) (No net change); IT, FS: 45 LTP, 48 LTD, 49 No (n = 142, No net change); IT, non-FS: 31 LTP, 10 LTD, 25 No (n = 66, net LTP)	Weight-dependent heterosynaptic plasticity (amplitude change correlated with initial PPR)	Presyn (PPR, CV)	Wistar rat P15–34 28–32°C

(Continued)

TABLE 1 | Continued

Reference Cortical region (connection #)	Layer cell type	Stim site/Input	Induction protocol	Blocker/Agonist in bath	Homosynaptic LTP, LTD, No (out of N cases)	Heterosynaptic	Mechanisms/Involved receptors/Ca++ source/Cascades	Pre/Post (measure)	Species Age rec T°
Huang et al. (2013) *visual, somatosensory cortex*	L2/3 FS PV+ non-FS SOM+	L4, below recording site, two bipolar electrodes	Pairing: synaptic stimuli before or after postsynaptic bursts 4 APs @100 Hz, x200@1 Hz; pre-then-post in one input, post-then-pre in the other input to the same cell; pre-post intervals: ±10, 25, 50 ms;	—	FS PV+: No changes in ctrl solution (95 ± 13%, *n* = 10 pre-then-post; 91 ± 6% *n* = 14 post-then-pre); LTD with α1-adrenoreceptors activated (methoxamine; 59 ± 5%, *n* = 6 and 51 ± 6%, *n* = 8 pre->post; 67 ± 7% *n* = 11 post->pre; 96 ± 4% *n* = 10 in un-paired); LTP with β-adrenoreceptors activated (isoproterinol; 132 ± 15%, *n* = 11 and 150 ± 25%, *n* = 9 pre->post; 130 ± 14% *n* = 12 post->pre; 102 ± 9% *n* = 14 in un-paired); STDP with both α1 and β agonists iso+met (136 ± 12% *n* = 11 pre->post and 72 ± 6% *n* = 11 post->pre at 10 ms; less at 25 ms, no at 50 ms); non-FS SOM+: similar, STDP in iso+met	No changes in un-paired, though 102±9% (SEM) *n* = 14 and 96±4% *n* = 10 might have included some LTP and LTD in individual experiments	FS PV+: Ca++ dependent, STDP in iso+met prevented by 10 mM BAPTA; NMDA-independent, APV did not prevent LTD in met, nor LTP in iso, nor STDP in iso+met; mGluR5 blocker MPEP prevented STDP in iso+met; preventing phosphorilation/trafficking of GluA1 prevented LTD, and both pre-post and post-pre pairing induced LTP; non-FS SOM+: NMDA-dependent, STDP in iso+met is prevented by APV	Postsyn (PPR)	Mouse P21–25
Kerkhofs et al. (2018) *medial prefrontal cortex*	L5 FS;	Bipolar electrode around dendrites	TBS: 5 stim @100 Hz x10, x3	—	Control solution: 7 LTP, 0 LTD, 3 No (*n* = 10); with adenosine A2R blocked by SCH58261: 0 LTP, 7 LTD, 3 No (*n* = 10)	—	Adenosine A2R availability controls the direction of plasticity, LTP/LTD	—	Wistar rat P35–46 32°C

Studies of synaptic plasticity in cortical neurons are sorted by the cortical region in which inhibitory neurons were recorded and origin of presynaptic fibers. Connection # corresponds to ***Figure 1****.*

Thus, initial studies demonstrate that CA1 interneurons can express long-term synaptic plasticity, which is calcium-dependent (Cowan et al., 1998; Wang and Kelly, 2001) but not input-specific (McMahon and Kauer, 1997; Cowan et al., 1998). Later research employing Ca^{2+} imaging has demonstrated that synaptically induced rises of $[Ca^{2+}]_i$ in aspiny dendrites do not spread much but are kept local by interneuron-specific mechanisms (Goldberg et al., 2003a; see section on calcium sources below for detail). Although the original rationale for a lack of input-specificity in interneurons because of lacking compartmentalization of calcium signals in their aspiny dendrites appeared not to be correct, experimental results demonstrating heterosynaptic plasticity (plasticity at unstimulated inputs) in inhibitory neurons remain valid. The issue of input specificity of plastic changes is further discussed in **Box 1** and below in the sections on calcium signals and heterosynaptic plasticity.

These studies also found high within-experiment heterogeneity of the outcomes of plasticity induction in interneurons. Cowan et al. (1998) report, for tetanized (homosynaptic) inputs, LTP in 10, LTD in 17, and no changes in eight experiments. At nontetanized (heterosynaptic) sites, the proportion was similar: nine inputs expressed LTP, 18 LTD, and eight did not change. McMahon and Kauer (1997) observed LTD in 32 out of 49 tested inputs, LTP in three and no changes in the remaining 14. Taube and Schwartzkroin (1987) report that, out of 12 basket cells tested, three expressed potentiation, three expressed depression, and in the remaining six cells, no change or a small increase of EPSP amplitude was observed. Thus, at the same type of connection, the same induction protocol could lead to different outcomes, including induction of plasticity of the opposing sign. This suggests that additional factors, either related to cell intrinsic predispositions of synapses for plasticity or heterogeneity among stimulated input fibers or diverse subclasses of recorded neurons contribute to determining the outcome of plasticity in interneurons.

These initial studies in CA1 inhibitory neurons also demonstrate that plasticity rules and mechanisms in interneurons can be: (a) different from those known for pyramidal cells, e.g., NMDA-independent (Perez et al., 2001; Wang and Kelly, 2001); and (b) different between the different types of inhibitory neurons (Perez et al., 2001). In fact, a difference in experimental conditions as well as cell-type specificity of plasticity mechanisms and heterogeneity of recorded subpopulations of interneurons could have contributed to discrepancies between the findings of the above studies, e.g., whether CA1 inhibitory neurons express predominantly LTD (McMahon and Kauer, 1997) or also LTP (Taube and Schwartzkroin, 1987; Cowan et al., 1998) or whether pairing weak synaptic stimuli with depolarization can induce plasticity (Perez et al., 2001; Wang and Kelly, 2001) or not (McMahon and Kauer, 1997).

Cell and Connection-Type Specificity of Plasticity at CA1 Interneurons

Indeed, further research revealed remarkable differences in the requirements for induction and mechanisms of plasticity in different types of interneurons. At Schaffer collaterals/commissural inputs to CA1 str. radiatum interneurons, pairing synaptic stimulation with depolarization induced LTP, which required an NMDAR-mediated $[Ca^{2+}]_i$ rise and was expressed postsynaptically (Lamsa et al., 2005). The LTP occurred in about half of studied neurons. In contrast, in excitatory inputs from collaterals of CA1 axons to str. oriens/alveus interneurons, neither pairing synaptic stimuli with depolarization nor high-frequency bursts of strong stimuli induced LTP (Lamsa et al., 2007). LTP in these cells could be induced by high-frequency burst stimulation only if the stimuli were weak or paired with hyperpolarization of the postsynaptic cell (Lamsa et al., 2007). Induction of LTP required a $[Ca^{2+}]_i$ rise *via* CP-AMPARs and was not prevented by blockade of NMDARs. Requirement of this form of plasticity for hyperpolarization during the induction is explained by the increase of calcium influx *via* CP-AMPA receptors at hyperpolarized potentials, which can then reach the threshold for triggering plasticity. Because of the opposite-to-Hebbian requirement for the induction (hyperpolarization instead of depolarization and firing), this form of plasticity was called "anti-Hebbian" (Lamsa et al., 2007).

Testing CP-AMPAR-dependent plasticity in other classes of CA1 inhibitory neurons with diverse location (str. pyramidale, radiatum, oriens), morphology (axo-axonic, basket, bi-stratified, ivy, Schaffer collateral-associated cells), and pattern of expression of characteristic proteins (parvalbumin PV, neuropeptide Y, somatostatin SST, cannabinoid receptors of type 1 CBR1, nitric oxide synthase NOS) revealed further diversity of plasticity rules in interneurons (Nissen et al., 2010; Szabo et al., 2012). With NMDA receptors blocked, high-frequency stimulation paired with hyperpolarization induced LTP in perisoma-targeting PV-positive cells and LTD in dendrite-targeting PV-positive cells (Nissen et al., 2010). In NOS-positive ivy cells and SST-positive bi-stratified oriens-lacunisum/moleculare (O-LM) neurons, LTP could be induced by theta-burst stimulation, but LTP was prevented if theta-burst stimulation was paired with depolarization (Szabo et al., 2012). No CP-AMPAR-dependent plasticity could be induced in Schaffer-collateral-associated cells or in CBR1-positive neurons by any of the above protocols. Notably, CBR1-positive neurons did not express plasticity even with unblocked NMDA receptors (Nissen et al., 2010).

Different plasticity mechanisms also may be associated with different network roles of inhibitory neurons. As described above, Schaffer collateral inputs to str. radiatum interneurons, which mediate feed-forward inhibition, express Hebbian-type, NMDAR-dependent LTP (Lamsa et al., 2005). Synapses made by collaterals of CA1 pyramidal neurons onto str. oriens/alveus interneurons mediating feedback inhibition express "anti-Hebbian" LTP, dependent on calcium influx *via* CP-AMPARs (Lamsa et al., 2007). At these feedback synapses, Hebbian-type LTP could still be induced by pairing theta-burst stimulation with depolarization, but to achieve the needed $[Ca^{2+}]_i$ rise, activation of mGluR1α was required (Topolnik et al., 2006). Moreover, plasticity

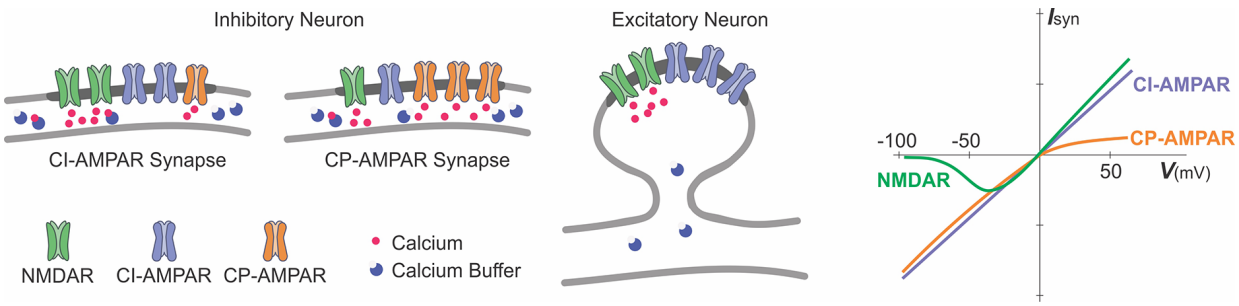
rules may be different at excitatory inputs that engage the same interneurons in either feed-forward or feedback inhibition. PV-positive interneurons in CA1 str. pyramidale receive feed-forward inputs from Schaffer collaterals and the perforant path as well as local feedback inputs from axon collaterals of local pyramidal neurons. In the feedback inputs, either Hebbian-type NMDAR-dependent or "anti-Hebbian" CP-AMPAR-dependent LTP could be induced, depending on whether synaptic stimulation was paired with depolarization (0 mV) or hyperpolarization (−90 mV). In contrast, only "anti-Hebbian" CP-AMPAR-dependent LTP could be induced at the feed-forward inputs (Le Roux et al., 2013).

Thus, the rules of induction and mechanisms of plasticity can be interneuron-type specific and even connection-type specific. In this context, "connection type" is defined by the identity of both presynaptic fibers and postsynaptic cells. Most illustrative here is the link between diverse plasticity rules and the diversity of sources of $[Ca^{2+}]_i$ rise, determined by the pattern of expression and subunit composition of NMDA, AMPA, and metabotropic glutamate receptors (see **Box 2**), which, in turn, correlates with the type of interneuron and connection.

Note that specificity of plasticity rules and mechanisms with respect to the type of interneuron and connection is not strict and precise. Observed variability could be due to intrinsic variability of synapses (e.g., of the ratios of NMDA/AMPA receptors and calcium permeable/impermeable AMPA receptors across synapses within the same type of connection) and also to differences in experimental conditions and plasticity induction protocols (see **Box 3** and **Table 1**) as well as animal lines and age and possible biases in sampling of highly heterogeneous inhibitory neurons. Regardless, an overall conclusion that plasticity in different types of interneurons and connections is mediated by different sets of mechanisms remains valid.

Plasticity of Excitatory Inputs to Interneurons in the CA3 Area of the Hippocampus and Dentate Gyrus

A unique experimental model to study cell-type and connection-type specificity of plasticity of excitatory inputs



BOX 2 | Glutamate receptor channels as sources of calcium influx.

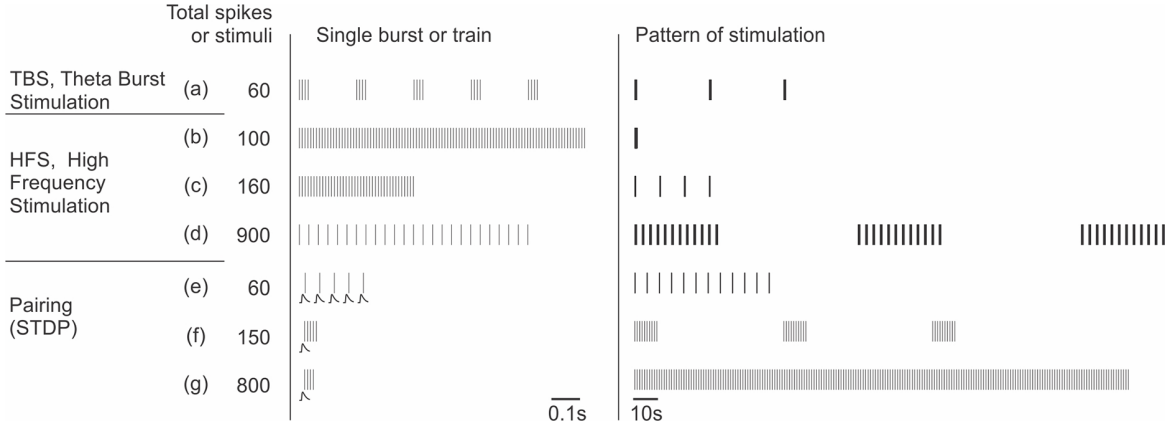
A schematic representation of excitatory glutamatergic synapses in inhibitory and excitatory neurons and a schematic plot of current-voltage relationships of glutamate-gated ionotropic channels.

Synapses at inhibitory neurons can express NMDA receptor channels (NMDAR), calcium-permeable AMPA-receptor channels (CP-AMPAR), and calcium-impermeable AMPA receptor channels (CI-AMPAR). The proportion of CP/CI AMPARs and of AMPARs/NMDARs varies across synapses (Laezza et al., 1999; Lei and McBain, 2002; Galván et al., 2008; Lalanne et al., 2016, 2018). At mossy-fiber synapses onto CA3 str. lucidum interneurons (Lei and McBain, 2002), expression of NMDARs is inversely related to the expression of CP-AMPARs, such that synapses with more NMDARs have less CP-AMPARs (left scheme), and vice versa, synapses with less NMDARs have more CP-AMPARs (middle). At other connections, no such correlation was found. In inhibitory neurons, fast kinetics of CP-AMPARs (Geiger et al., 1995; Jonas and Burnashev, 1995; Angulo et al., 1997), a small diameter of dendrites and high buffering capacity (blue circles) help to restrict the spread of the intracellular calcium.

Glutamatergic synapses at excitatory neurons are typically formed at dendritic spines (right scheme). At spine synapses, the canonical source of calcium entry that can trigger synaptic plasticity is through NMDARs, and the spike neck helps to restrict the spread of intracellular calcium.

Synaptic current and influx of calcium through the NMDARs and CP-AMPARs have distinct dependence on voltage (rightmost plot). Owing to a magnesium block at hyperpolarized potentials, opening of NMDAR channels requires both binding of glutamate to the receptor and depolarization to relieve the pore from magnesium block. This combination of requirements makes NMDAR a "coincidence detector" for coordinated presynaptic activity that supplies glutamate and postsynaptic activation that depolarizes the dendrite. CP-AMPARs are blocked at depolarized potentials by intracellular polyamines (Rozov and Burnashev, 1999). The polyamine block leads to a characteristic rectification of the current-voltage relationship of CP-AMPARs. Rectification of the voltage-current relationship of CP-AMPARs and magnesium block of NMDARs proved to be useful for a quick electrophysiological assessment of presence and relative contribution of CP-AMPARs and NMDARs to synaptic responses (e.g., Laezza et al., 1999; Lei and McBain, 2002; Galván et al., 2008).

Calcium permeability of fast AMPAR channels depends on their subunit composition, specifically, on the presence or absence of GluRB (GluR2) subunit. AMPARs at synapses in excitatory neurons contain an edited GluRB subunit with positively charged arginine at a particular position of the pore-forming segment, which prevents calcium ions from passing through the pore. Inhibitory neurons, however, can express AMPARs that lack a GluRB subunit and are permeable for calcium. Because AMPARs are heteromers, the ratio of CP to CI AMPARs depends on the level of expression of the edited GluRB subunit (Jonas et al., 1994; Geiger et al., 1995; Jonas and Burnashev, 1995; Koh et al., 1995; Angulo et al., 1997). Relative calcium permeability of AMPARs, characterized by the ratio of P(calcium)/P(monovalent ions) varies in different types of interneurons, e.g., 1.6 in dentate gyrus basket cells; 1.4 in dentate gyrus hillar neurons; 0.7 in inhibitory neurons from layer 4 of neocortex. For comparison, this ratio is <0.1 in excitatory neurons, such as L5 pyramids from neocortex; CA3 pyramids or granule cells from dentate gyrus (Geiger et al., 1995; Jonas and Burnashev, 1995); for NMDARs the ratio is >2.5 (Koh et al., 1995; Spruston et al., 1995).



BOX 3 | Stimulation protocols that induce plasticity in inhibitory neurons.

Representative stimulation protocols used to induce long-term plasticity in inhibitory neurons. Note that these protocols are same or similar to those used to induce plasticity in excitatory neurons (see, e.g., Chistiakova and Volgushev, 2009).

The "single burst or train" column in the middle shows timing of individual stimuli in afferent tetanization protocols or postsynaptic potentials and postsynaptic spikes in pairing protocols. "Pattern of stimulation" shows, at a compressed scale, timing of the whole protocol, each vertical bar representing a burst or a train from the middle column. Protocols are sorted by the total number of afferent stimuli or postsynaptic spikes for pairing protocols. Any of these protocols could be used either without injection of current into the recorded neuron, or in combination with depolarizing or hyperpolarizing current. Details of the protocols used in specific studies are given in **Table 1**.

Afferent tetanization protocols, theta-burst stimulation (TBS), and high frequency stimulation (HFS) typically use strong stimuli that evoke spikes in the postsynaptic neuron.

Theta-burst stimulation (TBS; a; from Perez et al., 2001) consists of short, high-frequency bursts of afferent stimuli (four stimuli at 100 Hz) repeated at 5 Hz. In the illustrated example, such trains are repeated three times.

High-frequency stimulation (HFS; b–d). A canonical form of HFS uses 100 stimuli at 100 Hz (b; from Taube and Schwartzkroin, 1987). Such 1 s trains can be repeated 2–4 times every 10 s. Shorter trains (c; from Cowan et al., 1998), or stimulation at lower frequency (30 Hz, as in d; from Alle et al., 2001) are also common.

Pairing protocols (e–g) consist of pairing subthreshold stimulation with postsynaptic spikes with specific, strictly defined relative timing to induce spike-timing dependent plasticity (STDP). Bursts of postsynaptic spikes, which are more effective at causing calcium influx into the cell, are typically used. In bursts of lower frequency, each presynaptic stimulus is paired with one postsynaptic spike (20 Hz, as in e; from Lu et al., 2007). Alternatively, each presynaptic stimulus can be paired with a high-frequency burst of postsynaptic spikes (100 Hz, as in f; from Chistiakova et al., 2019, and g; from Huang et al., 2013).

to interneurons is offered by the circuitry in the dentate gyrus and CA3 region of the hippocampus (**Figure 1**). Axons of dentate gyrus granular cells (mossy fibers) innervate, in addition to CA3 pyramidal neurons, inhibitory neurons in CA3 and in the dentate gyrus. Inhibitory neurons in the CA3 also receive excitatory inputs from recurrent collaterals of local CA3 pyramids and commissural fibers. Interneurons in the dentate gyrus also receive input from perforant path fibers originating in the entorhinal cortex. Thus, there are different types of interneurons innervated by the same mossy fibers as well as interneurons of the same type receiving inputs from clearly distinct sources.

Diverse Ca^{2+} Sources Contribute to Heterogeneity of Plasticity Rules and Mechanisms in CA3 Interneurons

An initial study found that tetanic stimulation of mossy fiber inputs to CA3 interneurons induced LTD in six out of nine cells (Maccaferri et al., 1998). Like canonical presynaptic LTP at mossy fiber inputs to CA3 pyramidal cells (Zalutsky and Nicoll, 1990), plasticity induction was NMDAR independent and did not require postsynaptic $[Ca^{2+}]_i$ rise, and expression was presynaptic. Remarkably, however, the outcome of plasticity was LTD rather than the LTP seen at pyramidal cells (Maccaferri et al., 1998). Further research (Lei and McBain, 2002; Galván et al., 2008, 2015) demonstrated that plasticity in CA3 interneurons was actually $[Ca^{2+}]_i$-dependent as it was blocked by fast calcium buffer BAPTA (in contrast to the slow buffer EGTA used in the Maccaferri et al., 1998 study), and revealed distinct sources for $[Ca^{2+}]_i$ rise and plasticity mechanisms in CA3 interneurons.

Mossy fiber synapses at str. lucidum interneurons contain calcium-permeable (CP) and calcium-impermeable (CI) AMPARs as well as NMDARs. The ratio of CP/CI-AMPAR expression covaried with the expression of NMDARs, forming a continuum from synapses with mostly CP-AMPARs and weaker and slower NMDAR-mediated components to synapses with mostly CI-AMPARs but a strong and fast NMDAR component (Bischofberger and Jonas, 2002; Lei and McBain, 2002). Calcium-dependent LTD induced by high-frequency stimulation was NMDAR-dependent and expressed postsynaptically at CI-AMPAR synapses but was NMDAR-independent and expressed presynaptically at CP-AMPAR synapses (Lei and McBain, 2004). Recent work suggests that induction of LTD at CP-AMPAR synapses involves release of BDNF from mossy fibers, which acts on postsynaptic TrkB receptors and triggers synthesis and release of endocannabinoids. Cannabinoids serve as a retrograde signal leading to reduction of glutamate release and, thus, presynaptic expression of LTD. When this signaling pathway was blocked or impaired, the proportion of LTD

cases became smaller, but notably, LTP could also be observed (Pan et al., 2019).

At mossy fiber CI-AMPAR synapses onto CA3 str. lacunosum/moleculare interneurons, associative LTP could be induced by pairing high-frequency tetanization with depolarization (Galván et al., 2008). LTP was NMDAR-independent but required $[Ca^{2+}]_i$ rise *via* L-type voltage-gated calcium channels (VGCCs). Blockade of additional calcium sources, such as mGluR1α receptors or calcium release from intracellular stores *via* IP3 receptor or ryanodine receptor-mediated cascades, resulted in induction of LTD instead of LTP. Expression of both LTP and LTD involved presynaptic mechanisms (Galván et al., 2008). Because induction of LTP at mossy fiber synapses was not accompanied by significant changes at simultaneously tested associative-commissural inputs ($95 \pm 16\%$ in $n = 11$ experiments), the authors concluded that LTP in str. lacunosum/moleculare interneurons was input-specific (Galván et al., 2008).

Synapses formed by axon collaterals of local pyramids onto CA3 str. radiatum and lacunosum/moleculare interneurons express CI-AMPARs, CP-AMPARs, and NMDARs, but the ratio of the CP/CI-AMPARs at a synapse did not correlate with the strength of NMDAR-mediated response component (Laezza and Dingledine, 2004). Induction of plasticity by afferent tetanization depended on the interaction between NMDARs, CP-AMPARs, and mGluR7s and the age of animals used for slice preparation (Laezza et al., 1999; Laezza and Dingledine, 2004; Galván et al., 2015). In slices from very young rat pups (P9–P12), the direction of plasticity at CP-AMPAR synapses was controlled by membrane potential during the tetanization. LTP was induced by tetanization at −30 mV or by pairing, but mostly LTD was observed after tetanization applied at 0 mV or −70 mV or with intracellular BAPTA. Blockade of NMDARs prevented induction of both LTP and LTD (Laezza and Dingledine, 2004). These results indicate that, at P9-P12, the bulk of calcium influx occurs *via* NMDARs and, when combined with the influx *via* CP-AMPARs, could provide $[Ca^{2+}]_i$ rise sufficient for triggering LTP. However, if influx *via* one or both sources is reduced or postsynaptic calcium is partially buffered, only the threshold for LTD induction is reached. In contrast to CP-AMPAR synapses, at P9–12, afferent tetanization at −30 mV did not induce plasticity in CI-AMPAR synapses.

In slices prepared from P10–P16 animals, plasticity at recurrent-collateral synapses also could be induced during NMDAR blockade: high-frequency stimulation induced LTD in CP-AMPAR-synapses, but LTP or no changes at CI-AMPA synapses. LTD at CP-AMPA synapses required $[Ca^{2+}]_i$ rise and activation of mGluR7 for the induction, and was expressed presynaptically (Laezza et al., 1999). The requirements for activation of distinct calcium sources for induction of plasticity further changed in older animals ($P35 \pm 5$). With NMDARs unblocked, afferent tetanization induced LTD at recurrent-collateral synapses equipped with CP-AMPARs and LTP at CI-AMPAR synapses. LTP at CI-AMPAR synapses could be prevented by intracellular BAPTA or hyperpolarization to −100 mV during the tetanization. With NMDA receptors blocked, LTD was induced instead (Galván et al., 2015).

The requirement of NMDAR activation for LTP induction at recurrent-collateral synapses containing CI-AMPARs stands in contrast to the requirements for LTP induction at CI-AMPAR-synapses made by mossy fibers to the same neurons. LTP at mossy fibers was NMDAR-independent (Galván et al., 2015) but required calcium influx *via* L-type VGCCs (Galván et al., 2008). Distinct calcium sources activated distinct intracellular cascades: LTP at recurrent-collateral synapses involved CaMKII-signaling but not PKA-signaling while LTP at mossy fiber synapses was not impaired by the blockade of CaMKII-signaling but involved PKA-signaling (Galván et al., 2015).

To summarize, comparison of plasticity at three types of connections to CA3 interneurons (**Figure 1**, connections 5, 6, 7) supports the notion of the dependence of plasticity rules and mechanisms on the type of connection and on the pattern of expression of glutamate receptors at the tested synapses. Note, however, that differences in experimental conditions and plasticity induction protocols may have added to the diversity of results (see **Table 1** and **Box 3**). For example, afferent tetanization of mossy fibers induced diverse forms of LTD in str. lucidum interneurons (Lei and McBain, 2002, 2004; Pelkey et al., 2005). The same tetanization but paired with depolarization of the postsynaptic cell could induce diverse forms of both LTP and LTD in str. radiatum and lacunosum/moleculare interneurons (Galván et al., 2008, 2015). Further research is needed to disentangle the role of variations in experimental conditions from the connection-specificity of plasticity mechanisms.

Age-dependence of plasticity mechanisms and requirements for specific sources mediating $[Ca^{2+}]_i$ rise for induction of plasticity in interneurons could be one further factor contributing to the variability of reported results. An emerging pattern is that, in very young animals, cooperative action of several sources is needed to rise $[Ca^{2+}]_i$ above the thresholds for plasticity induction. With maturation, individual sources become strong enough to provide $[Ca^{2+}]_i$ rise sufficient for the induction of plasticity. Because available data are sparse, this scenario is speculative.

Associative Plasticity of Mossy Fiber Inputs to DG Basket Cells Requires CP-AMPARs and mGluRs but Not NMDARs

Inputs from mossy fibers onto local interneurons in the dentate gyrus, PV-positive fast spiking basket cells, show bidirectional associative plasticity (Alle et al., 2001; Sambandan et al., 2010; Hainmüller et al., 2014). An "associative" induction protocol (burst frequency stimulation of mossy fibers paired with postsynaptic spikes) induced LTP in these cells while a "nonassociative" protocol (same burst frequency stimulation but paired with hyperpolarization preventing spikes) induced LTD (Alle et al., 2001; Hainmüller et al., 2014). Induction of LTP required $[Ca^{2+}]_i$ rise although it was attenuated only by high concentration of intracellular BAPTA, indicating a high capacity of intrinsic calcium buffers in these cells (Alle et al.,

2001). LTP was NMDAR-independent, but required activation of CP-AMPARs, which are abundant at mossy fiber synapses on dentate gyrus interneurons (Sambandan et al., 2010). LTP induction also required activation of group I metabotropic glutamate receptors and PKC (Alle et al., 2001; Hainmüller et al., 2014). In the presence of mGluR1/5 blockers in the bath, associative burst-frequency stimulation induced LTD instead of LTP (Hainmüller et al., 2014). Expression of LTP and LTD involved presynaptic mechanisms as indicated by changes of the failure rate, paired-pulse ratio, and coefficient of variation (Alle et al., 2001).

Interim Summary: Plasticity in the Hippocampus and Dentate Gyrus Interneurons

To summarize, research into plasticity in inhibitory neurons in the hippocampus showed that excitatory inputs to different types of inhibitory neurons express a multitude of forms and mechanisms of plasticity, including Hebbian and non-Hebbian-type plasticity at synapses activated during the induction (homosynaptic palsticity) as well as plastic changes at synapses that were not active during the induction (heterosynaptic plasticity; see below for detailed discussion).

Notably, the rules of induction and mechanisms of plasticity can be connection-type specific and determined by the properties and identity of both presynaptic fibers and postsynaptic cells. In the illustrative case of mossy fibers, canonical presynaptic LTP in CA3 pyramidal cells is purely presynaptic with the induction independent of postsynaptic calcium (Zalutsky and Nicoll, 1990). In contrast, at mossy fiber synapses formed on diverse types of interneurons, both LTP and LTD, with pre- or postsynaptic mechanisms of expression could be induced. Moreover, induction of plasticity invariably required postsynaptic rise of calcium (Laezza et al., 1999; Alle et al., 2001; Lei and McBain, 2002; Galván et al., 2008; Hainmüller et al., 2014), whereby the source of $[Ca^{2+}]_i$ rise and intracellular cascades leading to long-term plastic changes could be interneuron specific. For example, in dentate gyrus interneurons, LTP depends on CP-AMPARs (Sambandan et al., 2010), in interneurons from CA3 str. lacunosum/moleculare LTP depends on activation of L-type calcium channels and mGluR1-alpha (Galván et al., 2008), and in interneurons from CA3 str. lucidum, two different forms of LTD are induced depending on whether the tested synapses are equipped with a higher proportion of CP-AMPARs or with a stronger NMDAR-mediated response component (Bischofberger and Jonas, 2002; Lei and McBain, 2002, 2004). That latter example shows that plasticity mechanisms may be distinct even at synapses made at the same interneuron type by presynaptic fibers originating from the same source. One important consequence of the diversity of rules and mechanisms of plasticity is that the same pattern of activity may lead to different outcomes and even opposite-sign changes; e.g., in CA3 str. lacumosum/moleculare interneurons, tetanization of mossy fibers paired with postsynaptic depolarization leads to LTP at synapses equipped with CI-AMPARs but to no changes or LTD at CP-AMPAR synapses (Galván et al., 2008).

Plasticity of Excitatory Inputs to Inhibitory Interneurons in the Neocortex

The diverse forms of connection-specific plasticity observed in hippocampal interneurons provide a framework for interpretation of sparse data on plasticity of excitatory inputs to inhibitory interneurons of the neocortex. Neocortical networks add several layers of complexity to research into plasticity in

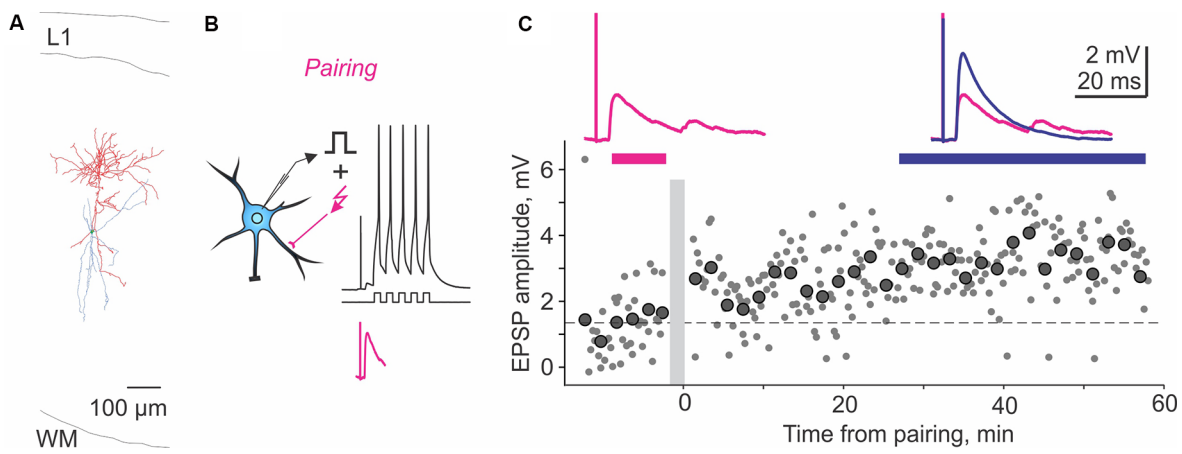


FIGURE 2 | Homosynaptic long-term potentiation (LTP) induced by pairing procedure in a Martinotti cell from rat visual cortex. **(A)** Reconstruction of the Martinotti cell with dendrites in blue and axon in red. L1, layer 1; WM, white matter. **(B)** A scheme of a pre-before-post STDP pairing protocol, in which presynaptic stimulation was followed with a 10-ms delay, by a burst of postsynaptic spikes evoked by five depolarizing pulses at 100 Hz. Lower trace (magenta) shows zoom in of the EPSP without postsynaptic spikes. The pairing procedure was repeated 30 times. **(C)** LTP induced in the paired input. Response amplitudes (small symbols show individual responses; large symbols—averages over 2 min) plotted against time after the pairing (gray vertical bar). Horizontal dashed line shows average response amplitude during control period. Traces show averaged responses during the indicated periods before (magenta) and after (blue) the pairing. Data from the cell shown in panel **(A)**. Modified with permission from Chistiakova et al. (2019).

inhibitory interneurons due to an even higher diversity of interneuron types than in the hippocampus (Markram et al., 2004; Ascoli et al., 2008; Gentet, 2012; Battaglia et al., 2013; Druckmann et al., 2013; Jiang et al., 2015; Tremblay et al., 2016), the area-specificity of function and circuitry (e.g., in somatosensory, visual, prefrontal areas) and high heterogeneity of connectivity within each area, which essentially requires recording from connected pairs of neurons or using other means of identification of stimulated presynaptic fibers for obtaining data that are clearly connection-specific. Therefore, for neocortical interneurons, plasticity rules often can be related only to the properties of the postsynaptic cells.

In somatosensory cortex slices from young rats (P13–16), different plasticity rules were found at connections made by L2/3 pyramids onto either low-threshold spiking (LTS) or FS cells (Lu et al., 2007). In connections to LTS cells, conventional spike-timing-dependent plasticity (STDP) was observed after repetitive pairing of bursts of presynaptic and postsynaptic spikes. Pre-before-post pairing induced LTP, and pre-after-post pairing induced LTD at short intervals (8 ms). Both LTP and LTD were NMDA-dependent and not sensitive to mGluR blockade. In contrast, in connections from pyramidal cells to FS interneurons, only LTD was induced by either pre-post or post-pre pairing at short intervals. LTD in FS cells did not require NMDARs, but was prevented by the blockade of mGluRs (Lu et al., 2007). Plasticity windows were narrow in both LTS and FS cells; neither LTP nor LTD was induced after pairing with 25 ms or longer delays.

In mouse visual and somatosensory cortex, distinct mechanisms of plasticity have been reported for FS vs. non-FS cells. In FS cells from layer 2/3, theta-burst stimulation of input fibers from layer 4 induced LTP (Sarihi et al., 2008), and a conventional STDP protocol applied in the presence of agonists of α1 and β adrenergic receptors could induce LTP and LTD (Huang et al., 2013). LTP and LTD were not impaired by NMDAR blockade, but were prevented by the blockade of mGluR5. The theta-burst-induced LTP also could be prevented by blockers of the PLC-IP3 cascade and release from internal Ca^{2+} stores. In non-FS cells from L2/3 theta-burst stimulation of input fibers from layer 4 could also induce LTP but in only 6 out of 17 experiments and of a smaller magnitude than in FS cells (Sarihi et al., 2008). Like in FS cells, STDP could be induced in SOM-positive non-FS cells in the presence of agonists of α1 and β adrenergic receptors (Huang et al., 2013). Plasticity in non-FS interneurons was also NMDAR-independent.

The following studies provide further evidence for plasticity in FS and non-FS interneurons but did not investigate its NMDA dependence. In the medial prefrontal cortex, theta-burst stimulation induced LTP in FS cells (Kerkhofs et al., 2018). In the visual cortex, repetitive pairing of presynaptic stimulation with bursts of postsynaptic spikes can induce long-term plasticity in both FS and non-FS cells from layers 2/3, 4, and 5 (Chistiakova et al., 2019). Pre-before-post pairing induced LTP in 5 out of 10 cells (**Figure 2**). Potentiation was significant also for the average of 10 paired inputs pooled together despite the fact that two of 10 cells expressed LTD. Notably, the pairing procedure also induced heterosynaptic LTP or LTD at inputs that were not stimulated during the pairing. However, because LTP and LTD at these inputs were about balanced, the average of all heterosynaptic inputs was not significantly different from control (Chistiakova et al., 2019). In the visual cortex, a form of age-dependent LTP induced by a mostly postsynaptic protocol has been described at unitary connections from star pyramids to layer 4 FS cells (Lefort et al., 2013). In reciprocally connected star pyramid–FS cell pairs, depolarization-induced bursts of high-frequency spikes in the FS neurons were combined with subthreshold depolarization of star pyramids. During this protocol, postsynaptic FS cells fired vigorously (∼200 spikes) while presynaptic star pyramids may generate 1–2 occasional spikes. This protocol induced robust LTP in FS cells from P22–23 animals but not in younger animals.

The only form of plasticity in interneurons reported so far that did not require the rise of postsynaptic $[Ca^{2+}]_i$ was described in SST-expressing interneurons in mouse somatosensory cortex (Chen et al., 2009). This special form of LTP was induced by a very strong theta-burst stimulation (120–200 bursts of five stimuli at 100 Hz) and required cAMP-PKA signaling but was not impaired by intracellular BAPTA (30 mM), blockade of NMDAR, or L-type calcium channels. LTP expression involved presynaptic mechanisms. Note that, unlike in other studies of neocortical interneurons, experiments in this study were performed at room temperature (see **Table 1**), and the induction protocol was extremely strong. Conventional theta-burst stimulation (40–60 bursts) or afferent tetanization at 100 Hz did not induce this form of LTP (Chen et al., 2009).

Because of the diversity of investigated neocortical areas and heterogeneity of inhibitory neurons and connections, these data provide only a sparse and patchy picture. However, results are consistent with the interpretation suggested by research on hippocampal interneurons. All excitatory connections to inhibitory neurons studied so far could express long-term plasticity, including Hebbian-type bidirectional plasticity. In all but one report, induction of long-term plasticity required rise of postsynaptic $[Ca^{2+}]_i$. Both NMDAR-dependent and NMDAR-independent forms of plasticity are present in neocortical interneurons (Lu et al., 2007; Sarihi et al., 2008; Huang et al., 2013). Even sparse available data provide evidence for cell-type specific rules and mechanisms of plasticity in neocortical interneurons (e.g., PV-positive vs. SOM-positive cells, Huang et al., 2013), and the only study that compared properties of two clearly defined connections revealed that plasticity rules could be connection-specific (synapses made by pyramids onto FS vs. LTS cells, Lu et al., 2007).

Modulation of Plasticity in Interneurons

Plasticity in inhibitory neurons is regulated by major neuromodulators, including acetylcholine, noradrenaline, adenosine, and glutamate (*via* mGluRs). Blockade of specific receptors to these neuromodulators could either prevent induction of plasticity altogether or mediate a switch between LTP and LTD.

Blockade of mGluR1/mGluR5s prevented induction of LTP in interneurons from CA1 str. oriens (Perez et al., 2001) and could switch the outcome of tetanization from LTP to LTD in interneurons from CA3 str. lacunosum/moleculare (Galván et al., 2008) and in the dentate gyrus (Hainmüller et al., 2014). In neocortical FS neurons from visual and somatosensory cortex blockade of mGluR5 prevented LTP induction by theta-burst stimulation (Sarihi et al., 2008) and prevented induction of LTP and LTD by an STDP protocol (Huang et al., 2013). Nonspecific blockade of mGluRs prevented paring-induced LTD at unitary connections from L2/3 pyramids to FS neurons in somatosensory cortex (Lu et al., 2007).

Cholinergic modulation of plasticity has been described in a subpopulation of SST-positive interneurons in CA1 str. oriens/alveus, which expresses calcium-permeable acetylcholine receptors (Jia et al., 2010; Griguoli et al., 2013). In these neurons, activation of nicotinic receptors was required for the induction of calcium-dependent, NMDAR-independent LTP by high-frequency tetanization paired with hyperpolarization. The two studies disagree on whether non-α7 nicotinic receptors (Jia et al., 2010) or α7 nicotinic receptors (Griguoli et al., 2013) mediated the calcium influx needed for LTP induction.

Requirement for activation of adrenergic receptors for the induction of bidirectional Hebbian-type plasticity by STDP protocol has been described in PV-positive FS and SOM-positive non-FS cells from the visual and somatosensory cortex (Huang et al., 2013). In both types of neurons, activation of β-adrenoreceptors was necessary for induction of LTP, and activation of α1-adrenoreceptors was necessary for induction of LTD. Without adrenergic agonists, no plasticity could be induced; in the presence of only β-adrenoreceptor agonists, only LTP and, in the presence of only α1 adrenoreceptor agonists, only LTD could be induced by both pre-before-post and pre-after-post pairing. With agonists of both α1 and β adrenergic receptors, canonical STDP was induced (Huang et al., 2013).

Modulation of plasticity by adenosine has been described for FS interneurons from prefrontal cortex (Kerkhofs et al., 2018). Theta-burst stimulation induced LTP in these interneurons in control conditions, but with adenosine A2 receptors blocked, the same stimulation induced LTD.

To summarize, major neuromodulatory systems are involved in regulation of plasticity in interneurons, and available data indicate that expression of distinct sets of neuromodulatory mechanisms may be among the factors that determine connection-specificity of plasticity rules.

CALCIUM SOURCES AND INTRACELLULAR DYNAMICS IN INTERNEURONS

Common Aspects of Calcium Signaling in Interneurons

One common condition for induction of diverse forms of plasticity at excitatory inputs to interneurons is the requirement for postsynaptic $[Ca^{2+}]_i$ rise. With an exception of LTP induced by strong TBS in SST+/PV- non-FS interneurons in the mouse somatosensory cortex (which was not blocked by intracellular BAPTA; Chen et al., 2009), all other forms of plasticity in interneurons for which the effect of buffering of postsynaptic calcium was tested report that induction of plasticity was prevented (Cowan et al., 1998; Laezza et al., 1999; Wang and Kelly, 2001; Lei and McBain, 2002; Lamsa et al., 2005, 2007; Jia et al., 2010; Hainmüller et al., 2014; Nicholson and Kullmann, 2014) or attenuated (Alle et al., 2001).

Differential Calcium Thresholds for LTP and LTD in Interneurons

Results of research into how plasticity in interneurons is affected by manipulation of $[Ca^{2+}]_i$ rise, e.g., by modifications of induction protocols or partial block of calcium sources, are compatible with the idea of differential $[Ca^{2+}]_i$ thresholds for induction of LTP and LTD. This hypothesis has been initially proposed for pyramidal neurons (Bienenstock et al., 1982; Lisman, 1989, 2001). It postulates that $[Ca^{2+}]_i$ has to rise to a certain threshold to induce LTD and to a yet higher level to induce LTP. One prediction of this hypothesis is that, by reducing $[Ca^{2+}]_i$ rise produced by an "LTP-protocol," e.g., by partial blockade of sources of calcium, it may induce LTD instead. Indeed, evidence from experiments in which diverse sources of $[Ca^{2+}]_i$ rise were manipulated supports this prediction. In CA3 str. radiatum interneurons, HFS applied at −30 mV induced LTP in control, but if $[Ca^{2+}]_i$ rise was reduced by intracellular BAPTA, LTD was induced (Laezza and Dingledine, 2004). In CA1 s.oriens/alveus interneurons, TBS paired with depolarization induced LTP if applied in control or with moderate reduction of $[Ca^{2+}]_i$ rise by blockade of either ERK or Srk or intracellular calcium release, but the same protocol induced LTD if $[Ca^{2+}]_i$ rise was reduced further by combined blockade of several of these sources (Topolnik et al., 2006). In mossy fiber inputs to dentate gyrus interneurons, associative burst-frequency stimulation induced LTP in control, but if calcium influx was reduced by the blockade of mGluRs1/5, LTD was induced instead (Hainmüller et al., 2014). Reduction of $[Ca^{2+}]_i$ rise could also result in a lower probability of LTP induction. In a subpopulation of SST-positive interneurons from CA1 str. oriens, HFS reliably induced LTP in control (n = 17 cells), but in only 6 out of 17 cells when $[Ca^{2+}]_i$ rise was reduced by blockade of calcium-permeable ACh receptors (Griguoli et al., 2013).

Notably, for some forms of plasticity in interneurons, the relation between the magnitude of $[Ca^{2+}]_i$ rise and induction of LTP or LTD could be different from that in pyramidal neurons, e.g., lacking the "LTD" window altogether (Le Roux et al., 2013) or even the inverse (whereby lower influx induces LTP and LTD occurring after higher rises). In FS interneurons from str. oriens of the CA1, subthreshold TBS leading to small amplitude Ca^{2+} transients induced LTP, but suprathreshold TBS leading to large supralinear Ca^{2+} signals in the dendrite-induced LTD (Camiré and Topolnik, 2014). Strong TBS could still induce LTP, if $[Ca^{2+}]_i$ rise is reduced and supralinear summation prevented by blocking calcium-dependent calcium release with CPA (Camiré and Topolnik, 2014). At mossy fiber synapses onto CA3 str. lucidum

interneurons, high-frequency stimulation applied during the blockade of NMDARs induced LTD in 70 out of 84 cells (no changes in the remaining 14). However, when calcium influx was reduced by blockade of TrkB receptors or in TrkB knockout mice plastic outcomes shifted toward potentiation. LTP was induced in 11, and LTD in 12 out of 42 cells (Pan et al., 2019).

Thus, induction of LTP and LTD in interneurons can be related to the magnitude of $[Ca^{2+}]_i$ rise; however, in a cell-type specific way. The relationship of the outcome of plasticity to $[Ca^{2+}]_i$ rise can be similar or even opposite to the "canonical" dependence of thresholds for LTD and LTP induction in pyramidal neurons. One factor contributing to the observed diversity could be localization of calcium sensors of induction mechanisms relative to the sources of calcium influx.

Calcium Signals Are Local in Aspiny Dendrites of Interneurons

Synaptically evoked calcium signals in dendrites of interneurons can be local despite the absence of "restricting" morphological structures, such as spines. Several mechanisms, common for diverse types of interneurons, contribute to keeping calcium signals local in aspiny dendrites (Goldberg et al., 2003a; Kaiser et al., 2004; Goldberg and Yuste, 2005). Glutamate receptors mediating calcium influx in interneurons have rapid kinetics. This is true both for calcium-permeable AMPA receptors and also for NMDARs, which have faster kinetics in interneurons than in pyramidal cells (Bischofberger and Jonas, 2002; Lei and McBain, 2002, 2004). Interneurons have relatively thin dendrites and typically high buffer capacity for calcium (Matthews et al., 2013; Matthews and Dietrich, 2015) due to expression of diverse calcium buffers, such as calbindin, calretinin, or parvalbumin, which are hallmarks of diverse types of inhibitory neurons (e.g., Nissen et al., 2010; Gentet, 2012; Szabo et al., 2012; Tremblay et al., 2016; Pelkey et al., 2017). A combination of these factors—fast kinetics of channels mediating calcium entry, high buffering capacity, and thin dendrites—allows restriction of the spread of synaptic calcium signals (**Box 2**). Indeed, calcium imaging demonstrates that local synaptic activation in smooth dendrites produces microdomains of $[Ca^{2+}]_i$ rise restricted to one or few micrometer (Goldberg et al., 2003a; Kaiser et al., 2004; Rozsa et al., 2004). Thus, lack of spines does not prohibit localized calcium rise and signaling and, therefore, does not prevent induction of input-specific plasticity.

Note that calcium signals, mediated by ligand-gated mechanisms, are restricted to one or few μm around the activated synapse during responses to moderate levels of activity. Strong episodes of activity would expand $[Ca^{2+}]_i$ rise as more synapses distributed over larger portions of dendrites are engaged. In addition, strong activity may lead to spillover of transmitter and activation of extrasynaptic receptors in a broader region, which is, however, not clearly defined for physiological conditions. Spillover may engage ligand-gated mechanisms also at nearby dendrites, including dendrites of other cells within the spillover area, but still within a local region around the activated synapses.

Despite the common aspects of calcium signaling considered above, specific calcium sources, dynamics, and thresholds for induction of plasticity in interneurons are highly diverse and can be cell-type and connection-type specific. Below we first describe sources of calcium rise grouped into: (a) synaptic and other ligand-gated mechanisms; and (b) nonsynaptic, voltage-gated mechanisms, such as back-propagating action potentials and voltage-gated calcium channels, and then consider how the interaction of diverse mechanisms determines calcium dynamics in interneurons.

Synaptic and Other Ligand-Gated Mechanisms Mediating Calcium Rise

Ligand-gated mechanisms contributing to the rise of $[Ca^{2+}]_i$ in interneurons include influx through NMDARs, calcium-permeable AMPARs, and calcium-permeable AChR channels and mechanisms coupled to mGluRs.

NMDAR and Calcium-Permeable AMPAR Channels

NMDAR channels represent a canonical source for the $[Ca^{2+}]_i$ rise that can trigger long-term plasticity in excitatory neurons (Bliss and Collingridge, 1993). In inhibitory neurons, expression of NMDARs, their contribution to the total calcium signal, and requirement for their activation for induction of plasticity is nonuniform. Typical ranges for NMDAR contribution to the calcium signal can be specific to distinct interneuron and connection types. In area CA1 of the hippocampus, quantitative immunogold labeling reveals that NMDARs, while consistently present at all spines on pyramidal cell dendrites, were found at low and variable density at dendrites of PV-positive interneurons with about 50% of dendrites lacking the label. Somata and dendritic shafts of SST-positive interneurons expressed highly variable density of NMDARs (Nyíri et al., 2003).

AMPARs in inhibitory interneurons can be calcium-permeable, depending on their subunit composition (Geiger et al., 1995; Jonas and Burnashev, 1995). AMPARs lacking the GluRB (GluR2) subunit have high permeability for calcium, fast kinetics, and are typically blocked by intracellular polyamines at positive potentials (Rozov and Burnashev, 1999). AMPARs containing edited GluRB subunit(s) have little calcium permeability, slow kinetics, and are not sensitive to polyamine block (see **Box 2**). The proportion of CP to CI AMPARs can differ systematically between cells of distinct types and can be different even at synapses originating from same presynaptic cells, e.g., in mossy fiber synapses to CA3 interneurons (Lei and McBain, 2002; Galván et al., 2008). In the neocortex, synapses made by pyramidal cells onto PV-positive basket cells express CP-AMPARs, but those at SST-positive Martinotti cells do not (Lalanne et al., 2016).

Although the identity of the postsynaptic cell is certainly the major determinant of the expression of postsynaptic receptors, their composition could be also connection-type specific, i.e., correlate with the source of presynaptic fibers that make synapses on the same postsynaptic neuron. Perisomatic inhibitory neurons in the dentate gyrus typically express more CP-AMPARs and less NMDARs at synapses received from mossy

fibers, but more CI-AMPARs and more NMDARs at synapses made by the perforant path fibers (Sambandan et al., 2010; see **Box 2**). At mossy fiber synapses onto CA3 interneurons, expression of CP-AMPARs and NMDARs was inversely related: synapses with more CP-AMPARs contained less NMDARs, and vice versa, synapses with less CP-AMPARs but more CI-AMPARs contained more NMDARs (Lei and McBain, 2002). At recurrent collateral synapses made on these same cells by axon collaterals of local pyramids, no such correlation was found (Laezza et al., 1999; Laezza and Dingledine, 2004). In both mossy fiber and recurrent collateral connections to CA3 interneurons, more synapses were equipped with CI-AMPARs than with CP-AMPARs (Galván et al., 2008, 2015).

These results illustrating connection-specificity of the expression of CI-AMPARs, CP-AMPARs, and NMDARs, are in accordance with the NMDAR or CP-AMAPR-dependent mechanisms of plasticity revealed at respective synapses. Further support to the link between NMDAR and CP-AMPAR-mediated calcium influx on the one hand and specific forms of plasticity on the other comes from calcium imaging studies.

In the mouse visual cortex, in calretinin-positive irregular-spiking and adapting interneurons, NMDARs were the major source of calcium during synaptic stimulation. In these non-FS cells, blockade of NMDARs completely eliminated calcium signal in the dendrites or reduced it to <10% of control. In contrast, in PV-positive FS cells, blockade of NMDARs had variable effect on $[Ca^{2+}]_i$ rise, ranging between a complete block in 2 out of 17 cells, a negligible <10% reduction in two other, and intermediate reduction in the remaining 13 cells. The remaining calcium signal in FS cells could be blocked by AMPAR-antagonist DNQX or a selective CP-AMPAR blocker philanthotoxin and was, thus, mediated by CP-AMPARs (Goldberg et al., 2003c). Major contribution of NMDARs to $[Ca^{2+}]_i$ rise in all non-FS but in only few FS cells parallels results on NMDAR-dependent plasticity in non-FS neurons, and NMDAR-independent plasticity in FS neurons from the visual and somatosensory cortices, considered above (Lu et al., 2007; Sarihi et al., 2008; Huang et al., 2013).

In CA1 interneurons from str. oriens-alveus, including FS basket and bistratified neurons, synaptically evoked calcium signals were mediated predominantly by CP-AMPARs in 8 out of 14 cells, and predominantly by NMDARs in 6 out of 14 cells (Topolnik et al., 2005; Camiré and Topolnik, 2014). These results are paralleled by reports that, in most of these neurons "anti-Hebbian" LTP can be induced during NMDAR blockade (Lamsa et al., 2007), and either Hebbian or non-Hebbian LTP with NMDARs unblocked (Le Roux et al., 2013). In perisomatic interneurons in the dentate gyrus CP-AMPARs were the major source of calcium signal during burst-stimulation (Hainmüller et al., 2014), corresponding to the CP-AMPAR-dependent LTP in these cells (Sambandan et al., 2010).

An interesting aspect of calcium signaling *via* NMDARs and CP-AMPARs comes from a study of sparsely spiny FS interneurons. In these cells, a relative contribution of NMDARs and CP-AMPARs to the total calcium influx depends on whether the synapse is located on a spine or on a dendritic shaft. Both types of synapses may be equipped with both NMDARs and CP-AMPARs, but the proportion of NMDARs is higher at spines, and proportion of CP-AMPARs is higher at synapses made on dendritic shafts (Sancho and Bloodgood, 2018).

To summarize, cortical interneurons express plasticity forms that depend on calcium influx *via* NMDARs, CP-AMPARs, or both. Because of the different voltage dependence, calcium influx through CP-AMPAR and NMDAR channels is maximized in different ranges of the membrane potential (**Box 2**). Calcium influx through CP-AMPARs increases with hyperpolarization, and under physiological conditions is maximal at or below the resting potential, for example, when CP-AMPAR activation coincides with strong inhibition. Calcium influx through NMDARs is maximal at depolarized potentials around −50 mV to −30 mV, when the magnesium block is relieved, e.g., by strong excitation. This creates differential requirements for the induction of NMDAR or CP-AMPAR dependent plasticity (see section on plasticity and **Table 1**). Importantly, differential voltage-dependence of NMDARs and CP-AMPARs also expands the range of membrane potentials at which plasticity can be induced.

Metabotropic Glutamate Receptors (mGluRs)

Several types of metabotropic glutamate receptors (mGluRs) contribute to $[Ca^{2+}]_i$ rise and induction of plasticity in interneurons. In CA1 str. oriens/alveus interneurons, local puffs of agonists of group I or group I/II mGluRs could produce an increase of $[Ca^{2+}]_i$ in the dendrites (Gee et al., 2001; Topolnik et al., 2006). Calcium signals had either fast or slow kinetics and were mediated by distinct mechanisms. Fast signals were mediated by mGluR1α leading to activation of transient receptor potential (TRP) channels and release from internal calcium stores. Slow calcium signals were mediated by mGluR5 and exclusively by release from internal stores. Because mGluRs can be recruited by high-frequency or theta-burst stimulation (Topolnik et al., 2005, 2006), they could contribute to $[Ca^{2+}]_i$ rise needed for plasticity induction. Indeed, activation of mGluR1α was necessary for induction of LTP in str. oriens neurons (Perez et al., 2001; Topolnik et al., 2006; Griguoli et al., 2013). Activation of mGluR5 was necessary for induction of LTP in L2/3 interneurons from visual cortex (Sarihi et al., 2008), LTD at connections between L2/3 pyramids and FS interneurons in somatosensory cortex (Lu et al., 2007), and timing-dependent LTP and LTD by STDP protocol in PV-positive interneurons from visual and somatosensory cortices (Huang et al., 2013). mGluRs could also play a role of a switch from LTD to LTP, so that activation of mGluRs combined with other sources of calcium could produce $[Ca^{2+}]_i$ rise needed to induce LTP while, without mGluR activation, only the calcium threshold for LTD induction is reached. Indeed, at mossy fiber synapses onto perisomatic inhibitory neurons in the dentate gyrus (Hainmüller et al., 2014) and CA3 str. lacunosum/moleculare interneurons (Galván et al., 2008), LTP was induced in control conditions, but with group I mGluRs blocked, LTD was induced instead.

Calcium-Permeable Acetylcholine Receptors (CP-AChRs)

A subset of interneurons in CA1 str. oriens expresses calcium-permeable acetylcholine receptors (Jia et al., 2010; Griguoli et al., 2013). These cells were bistratified oriens-lacunosum/moleculare (O-LM) neurons and expressed SST and NPY but neither PV nor CB (Jia et al., 2010). With glutamatergic and GABA-ergic synaptic transmission blocked, cholinergic responses in these interneurons could be evoked by application of nicotine or synaptic stimulation (Jia et al., 2010; Griguoli et al., 2013). Rise of $[Ca^{2+}]_i$ in response to nicotine was mediated by both CP-AChRs and voltage-gated calcium channels (Jia et al., 2010). Calcium influx *via* CP-AChRs was necessary for the induction of NMDAR-independent "anti-Hebbian" LTP. Two studies diverge in identifying the specific subtype of nicotinic cholinergic receptors involved as non-α7 nAChRs (Jia et al., 2010) or α7 nAChRs (Griguoli et al., 2013), which could be due to the use of rats vs. mice for experiments.

Interim Summary: Ligand-Gated Mechanisms of Calcium Rise

To summarize, a variety of ligand-gated mechanisms mediate $[Ca^{2+}]_i$ rise in interneurons: NMDARs, calcium permeable AMPAR and AChRs channels, and metabotropic glutamate receptors. The set of mechanisms expressed at a synapse is naturally determined by the identity of the postsynaptic neurons (cell-specific); however, these sets also can be systematically different at synapses made at the same neuron by axons originating from different sources (connection-specific). At individual synapses, the contribution of diverse mechanisms to the total $[Ca^{2+}]_i$ rise vary markedly around the mean "connection-specific" values. Moreover, $[Ca^{2+}]_i$ rise at synapses of the same interneuron may be mediated by different combinations of ligand-gated calcium sources (Topolnik et al., 2005; Camiré and Topolnik, 2014; Sancho and Bloodgood, 2018).

Nonsynaptic Mechanisms of Calcium Rise: Back-propagating Action Potentials and Voltage-Gated Calcium Channels (VGCCs)

Nonsynaptic mechanisms can mediate $[Ca^{2+}]_i$ rise that is not restricted to the dendrites contacted by activated synapses but can involve dendritic branches and, at maximum, the whole dendritic tree of the activated cell.

Interneurons, like pyramidal cells, express in their dendrites voltage-gated sodium and calcium channels, which support back-propagating action potentials (bAPs) and calcium influx (Martina et al., 2000; Kaiser et al., 2004). Dendritic calcium signals produced by bAPs have been reported for all interneurons studied so far, e.g., bitufted interneurons from L5 of visual cortex (Kaiser et al., 2004); multipolar FS PV-positive interneurons, bipolar irregular spiking CR-positive interneurons, and a heterogeneous group of adapting interneurons from L2/3 of visual cortex (Goldberg et al., 2003a,b; Sancho and Bloodgood, 2018); LTS SST-positive Martinotti cells from L5 of visual and somatosensory cortex (Goldberg et al., 2004); interneurons from str. radiatum of CA1 region in the hippocampus (Rozsa et al., 2004; Evstratova et al., 2011); CA1 oriens/alveus interneurons (Topolnik et al., 2009; Camiré and Topolnik, 2014); perisomatic interneurons from the dentate gyrus (Hainmüller et al., 2014). In interneurons, dendritic calcium signals are smaller and slower than in pyramidal cells. Back-propagation of APs into distal dendrites and related increase of $[Ca^{2+}]_i$ requires sodium channels and blockade of sodium channels with TTX typically restricts calcium signals to ∼100 μm from the soma (Goldberg et al., 2003a; Kaiser et al., 2004; Evstratova et al., 2011). One remarkable exception here is active propagation of bursts of spikes into dendrites of LTS Martinotti cells from L5 of visual and somatosensory cortex. In burst mode, these cells can produce regenerative TTX-independent calcium spikes that propagate throughout the dendritic tree, and the amplitude of dendritic $[Ca^{2+}]_i$ rise can even increase with distance from the soma (Goldberg et al., 2004).

In all types of interneurons tested, bursts of spikes propagate into distant sites more effectively than single action potentials and evoke stronger calcium signals. The amplitudes of bAPs and related calcium signals in dendrites typically decay with distance, nonuniformly in neurons of diverse types. In CA1 oriens/alveus interneurons, simultaneous recordings from the soma and dendrites at distances up to ∼100 μm revealed little decay of bAP amplitudes, which remained at 90% or higher of the somatic APs in most cells. The decay was similarly small for the first and the last AP in trains evoked by 100-ms pulses (Martina et al., 2000). In bitufted interneurons in L2/3 of the somatosensory cortex of rats, bAPs recorded at distances up to 50 μm from the soma had amplitudes above ∼80% of the somatic (Kaiser et al., 2004). Calcium imaging demonstrated that bursts of bAPs can evoke in these cells dendritic $[Ca^{2+}]_i$ rises even at the maximal measured distance of ∼400 μm. $[Ca^{2+}]_i$ rises in the distal dendrites, >200 μm from the soma, could have an amplitude comparable to that near the soma or be attenuated to ∼20%–30% (Kaiser et al., 2004). In interneurons from mouse visual cortex, bAP-evoked calcium signals decayed faster with distance. In FS PV-positive interneurons with multipolar dendrites, irregular spiking CR-positive cells with bipolar morphology, and a heterogeneous group of interneurons with adapting firing pattern, the amplitude of calcium signals at >100 μm was about 30% of the amplitude close to the soma (Goldberg et al., 2003b). Backpropagation of APs and related calcium signals in these cells was restricted by activation of potassium channels. With potassium and sodium channels blocked, long depolarization pulses induced strong calcium signals that did not attenuate with distance, indicating that voltage-gated calcium channels in these cells could support calcium influx throughout the dendritic tree (Goldberg et al., 2003a). In several types of interneurons from mouse hippocampus CA1, including basket and Schaffer-collateral associated cells from str. radiatum and basket and bistratified cells from str. oriens/alveus, calcium signals induced by bursts of bAPs attenuated below detection level at ∼150 μm from the soma (Evstratova et al., 2011; Topolnik, 2012; Camiré and Topolnik, 2014). An opposite situation with calcium signals increasing with distance from the soma has been reported for CA1 str. radiatum interneurons. The increment of bAP-evoked calcium signals measured in distal

dendrites up to about 150–160 μm from the soma could be due to the small diameter of distal dendrites (Rozsa et al., 2004). Active propagation of TTX-resistant calcium spikes in LTS Martinotti cells from L5 of the neocortex (Goldberg et al., 2004), considered above, represents another example of a nondecremental spread of calcium signal over the whole dendritic tree.

Calcium influx during bAPs is primarily mediated by voltage-gated calcium channels and amplified by release from internal stores. Application of nonselective blockers or a cocktail of channel-type selective blockers of VGCCs reduced calcium signals to 10%–15% (Goldberg et al., 2003a; Rozsa et al., 2004; Topolnik et al., 2009; Evstratova et al., 2011). In interneurons of different types, distinct sets of VGCCs may mediate calcium signals. In CA1 str. radiatum, bAP-evoked calcium signals were mediated by a combination of L-, T-, and P/Q- type VGCCs in basket cells, but by L- and T-type, with negligible contribution of P/Q channels, in Schaffer collateral-associated cells (Evstratova et al., 2011).

Interaction of Factors Determining Calcium Dynamics in Interneurons

Ultimately, dynamics of $[Ca^{2+}]_i$ in interneurons is determined by the interaction between multiple ligand-gated and voltage-gated sources of calcium influx described above as well as additional factors, such as calcium release from internal stores (e.g., Goldberg et al., 2003a; Topolnik et al., 2009; Evstratova et al., 2011; Camiré and Topolnik, 2014; Camiré et al., 2018) and internal calcium buffering and extrusion (Goldberg et al., 2003a; Rozsa et al., 2004; Evstratova et al., 2011; Matthews et al., 2013; Matthews and Dietrich, 2015; Chamberland et al., 2019).

Back-propagating APs, in addition to causing calcium influx *via* activation of VGCCs, can bidirectionally modify dendritic calcium signals produced by ligand-gated mechanisms. Back-propagating APs enhance NMDAR-mediated calcium signals in SST-positive and PV-positive interneurons from L2/3 of the visual cortex (Kaiser et al., 2004; Sancho and Bloodgood, 2018), similarly to the canonical mechanism of detection of coincident EPSPs and postsynaptic spikes in pyramidal neurons (Magee and Johnston, 1997; Markram et al., 1997). Also, mGluR mediated calcium signals in the dendrites of perisomatic inhibitory neurons in the dentate gyrus were enhanced by bAPs (Hainmüller et al., 2014). In contrast, $[Ca^{2+}]_i$ rise mediated by the CP-AMPARs is reduced by the spikes because of the decreasing driving force and eventual polyamine block at depolarized potentials (Rozov and Burnashev, 1999; Hainmüller et al., 2014; Sancho and Bloodgood, 2018). In FS interneurons from hippocampal CA1, strong stimulation of multiple presynaptic fibers can lead to supralinear summation of CP-AMPAR-mediated calcium signals due to calcium release from internal stores (Camiré and Topolnik, 2014; Camiré et al., 2018). Release from internal stores could also amplify calcium signals evoked by bAPs in several types of CA1 interneurons: CCK-positive basket cells and Schaffer collateral-associated cells from str. radiatum and interneurons from str. oriens/alveus (Topolnik et al., 2009; Evstratova et al., 2011).

One important factor that determines calcium dynamics in interneurons is high buffering capacity (Goldberg et al., 2003a; Rozsa et al., 2004; Evstratova et al., 2011). Expression of diverse calcium buffers, such as calbindin, calretinin, or parvalbumin, in neuron type-specific combinations (e.g., Nissen et al., 2010; Gentet, 2012; Szabo et al., 2012; Tremblay et al., 2016; Pelkey et al., 2017) results in marked differences between interneurons in calcium binding capacity (reviewed in Mattews et al., 2013; Matthews and Dietrich, 2015). The relation between calcium buffering capacity and calcium dynamics has been demonstrated in CA1 str. radiatum interneurons: calcium signals evoked by bAPs are larger and faster in basket cells with lower calcium buffering capacity than in Schaffer collateral-associated cells with higher capacity of calcium buffers (Evstratova et al., 2011). Type-specific differences in calcium buffering set distinct temporal and spatial restrictions on $[Ca^{2+}]_i$ rise and integration of calcium signals.

Sets of mechanisms mediating calcium influx while exhibiting a certain degree of specificity with respect to the type of interneurons and connections, may vary across cells and connections of the same type as discussed above. Moreover, synapses at the same neuron may express diverse sets of sources for $[Ca^{2+}]_i$ rises. Complementary sets of mechanisms mediating calcium influx at two dendritic locations of the same neuron had been clearly demonstrated in CA1 str. oriens interneurons. Calcium influx in response to glutamate puffs at one dendritic location was mediated almost exclusively by mGluRs with negligible contribution of NMDARs, AMPARs, and VGCCs while, at another location on the same cell, calcium responses were mediated by NMDARs, AMPARs, and VGCCs, and sequential blockade of these sources gradually reduced and eventually eliminated calcium responses (Topolnik et al., 2005). In sparsely spiny PV-positive interneurons from L2/3 of the visual cortex, synapses located on spines and on dendritic shafts both express CP-AMPARs and NMDARs, but the proportional contribution of NMDARs to calcium response in spines was about two times higher than at dendritic synapses (Sancho and Bloodgood, 2018).

To summarize, the requirement for $[Ca^{2+}]_i$ rise is one common condition for induction of long-term plasticity in inhibitory neurons. Multiple sources converge to contribute to the dynamics of intracellular calcium that ultimately determines whether and which intracellular mechanism(s) that may lead to long-term plasticity will be triggered. Manipulations that change the dynamics of intracellular calcium or the availability of intracellular cascades triggered by calcium rises may change the outcome of plasticity induction, e.g., between LTP and LTD.

HETEROSYNAPTIC PLASTICITY OF EXCITATORY INPUTS TO INHIBITORY NEURONS

Nonsynaptic mechanisms can produce $[Ca^{2+}]_i$ rise to the thresholds necessary to induce long-term plasticity not only at activated synapses, but also at nonactivated synapses, leading to heterosynaptic plasticity. By definition, heterosynaptic plasticity refers to changes at synapses that were not presynaptically activated during the induction protocol (**Box 1**). Initial studies

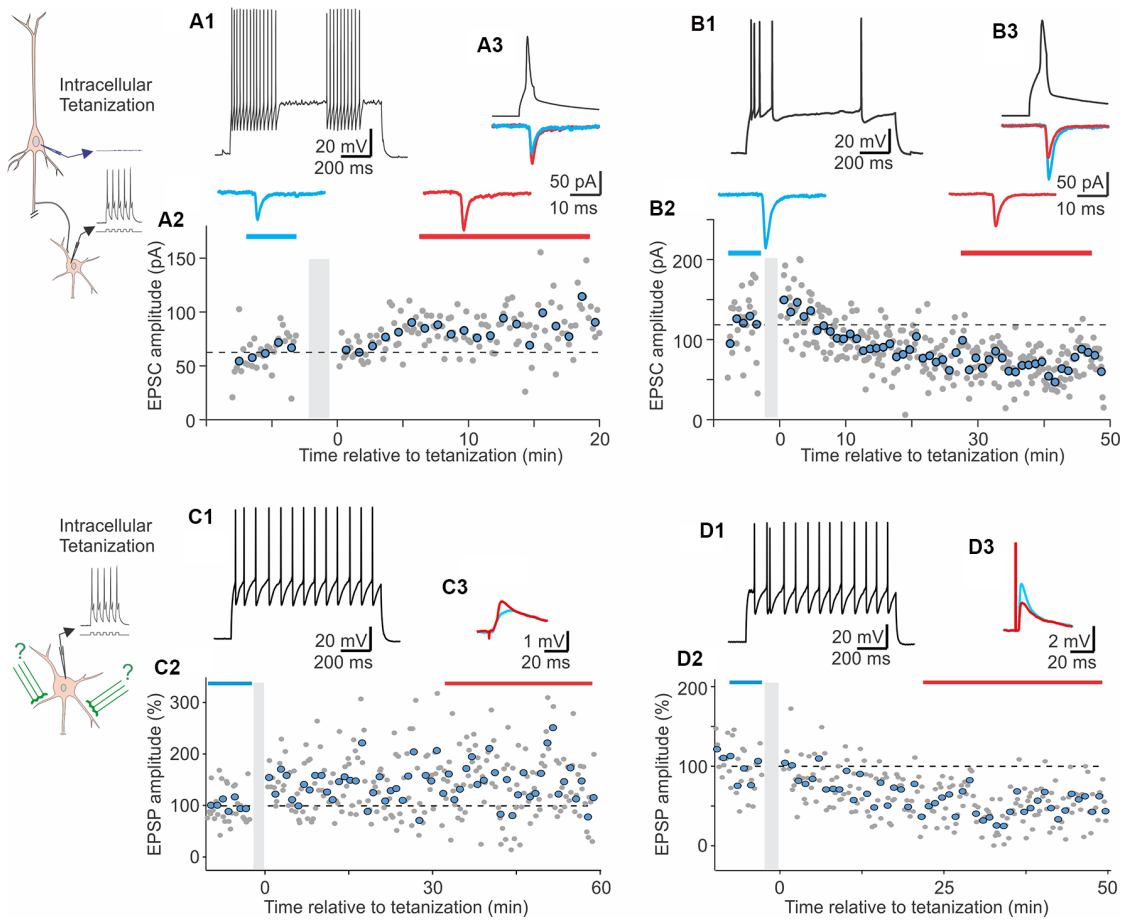


FIGURE 3 | Heterosynaptic plasticity induced by intracellular tetanization in unitary connections from pyramidal neurons to inhibitory interneurons **(A1–A3, B1–B3)** and in pharmacologically isolated excitatory inputs to inhibitory neurons **(C1–C3, D1–D3)** in slices from rat visual cortex. Insets on the left show schemes of the intracellular tetanization experiment. Intracellular tetanization consisted of 30 bursts of five spikes evoked in the postsynaptic cell by short depolarizing pulses (5 ms, 100 Hz) without presynaptic stimulation. Note that, in experiments with unitary connections, absence of spikes in presynaptic pyramidal neurons was verified. In experiments with pharmacologically isolated EPSPs, 50 μm PTX was present in the extracellular medium throughout the recording. **(A1–A3)** LTP at unitary connection from a pyramidal cell to a fast-spiking (FS) inhibitory neuron from layer 3. **(A1)** Firing pattern of the postsynaptic FS neuron in response to a 1 s depolarizing pulse. **(A2)** Time course of unitary EPSC amplitude changes. Time of intracellular tetanization is indicated by vertical gray bar. Gray circles are individual amplitudes; larger blue circles are averages over 1 min. Horizontal dotted line shows mean response amplitude before tetanization. Averaged EPSCs are shown from the indicated periods before and after tetanization. **(A3)** Superimposed averaged EPSCs from **(A2)**, together with an average of presynaptic spikes. **(B1–B3)** Long-term depression (LTD) of unitary EPSCs in a non-FS neuron from layer 3. **(B1)** Firing pattern of the postsynaptic non-FS neuron. **(B2)** Time course of unitary EPSC amplitude changes and averaged responses before and after tetanization. Same conventions apply as in **(A2)**. **(B3)** Superimposed averaged EPSCs from **(B2)**, and an average of presynaptic spikes. **(C1–C3)** LTP of pharmacologically isolated EPSPs in FS neuron. **(C1)** Firing pattern of the FS neuron. **(C2)** Time course of EPSP amplitude changes. Time of intracellular tetanization is indicated by vertical gray bar. **(C3)** Superimposed averaged EPSPs from the periods before and after intracellular tetanization indicated on the time course. **(D1–D3)** LTD of pharmacologically isolated EPSPs in FS neuron. **(D1)** Firing pattern of the FS neuron. **(D2)** Time course of EPSP amplitude changes. **(D3)** Superimposed averaged EPSPs from the periods indicated on the time course (modified with permission from Chistiakova et al., 2019).

of heterosynaptic plasticity in interneurons were motivated by the fact that interneurons have no or few spines on their dendrites with a majority of synapses made on dendritic shafts. Because spines restrict diffusion of molecules and ions, including calcium, the idea behind these experiments was that spread of intracellular calcium from active synapses along aspiny dendrites would facilitate induction of heterosynaptic plasticity at other, nonactivated synapses (McMahon and Kauer, 1997; Cowan et al., 1998). Indeed, in CA1 interneurons in the hippocampus, afferent tetanization induced LTD that could "spread" to nonactivated synapses (McMahon and Kauer, 1997) or induce plastic changes that lack input-specificity with LTP, LTD, or no changes occurring in both homosynaptic and heterosynaptic pathways in all possible combinations (Cowan et al., 1998). While the initial premise for lack of input specificity appeared to be wrong (aspiny dendrites do possess mechanisms that keep synaptically evoked $[Ca^{2+}]_i$ rises local; Goldberg et al., 2003a; Kaiser et al., 2004; Goldberg and Yuste, 2005), the above studies provide clear experimental evidence for heterosynaptic plasticity in hippocampal interneurons.

A large volume of subsequent work on interneurons from the hippocampus and neocortex reported only input-specific plasticity restricted to the synapses activated during the induction but no heterosynaptic changes (e.g., Lamsa et al., 2005, 2007; Pelkey et al., 2005; Galván et al., 2008, 2015; Sambandan et al., 2010; Huang et al., 2013; Le Roux et al., 2013; Nicholson and Kullmann, 2014). Note, however, that most of this research was aimed at in-depth analyses of specific forms of homosynaptic plasticity, and experimental conditions were optimized for the induction of these specific plasticity forms, e.g., induction protocols applied at hyperpolarized membrane potentials to maximize calcium influx *via* CP-AMPARs, and/or recordings were made with cesium-based intracellular solution and added sodium channel blocker QX-314 for better voltage control. Such experimental conditions, by impairing dendritic voltage-gated mechanisms and modifying calcium dynamics in the dendrites, might have impaired the induction of plasticity at heterosynaptic sites.

Calcium imaging shows that, in all interneurons studied so far, bAPs can propagate and evoke calcium signals in proximal dendrites, and in some types of interneurons reach distal parts of the dendritic tree (Rozsa et al., 2004) or even induce global dendritic calcium spikes, e.g., in LTS cells from L5 of the neocortex (Goldberg et al., 2004). Propagation of APs in a dendrite can be enhanced by depolarization produced by activation of synapses on that dendrite (for review see Goldberg and Yuste, 2005) or by downregulation of potassium channels that normally restrict backpropagation of APs (Goldberg et al., 2003b). Bursts of bAPs activate VGCCs that are present throughout the dendritic tree (Goldberg et al., 2003b). $[Ca^{2+}]_i$ rise mediated by VGCCs can be further amplified by release from internal stores (Topolnik et al., 2009; Evstratova et al., 2011). Combined action of these nonsynaptic mechanisms may rise $[Ca^{2+}]_i$ to the threshold for induction of long-term plasticity also at heterosynaptic sites within a dendritic branch that is currently most active or over broader regions of the dendritic tree.

The above scenario predicts that long-term plasticity in inhibitory neurons could be induced by strong postsynaptic activity without presynaptic activation. Indeed, in regular firing interneurons from CA1 region of the hippocampus, LTP could be induced by trains of postsynaptic spikes (about 600 APs) evoked by depolarizing pulses without presynaptic activity (Nicholson and Kullmann, 2014, 2017). LTP induced by AP trains shared common mechanisms of induction and expression with CP-AMPAR-dependent, NMDAR-independent LTP induced by afferent tetanization. Induction of LTP by either protocol was impaired by specific blockers of T-type calcium channels, indicating that T-channels contributed significantly to calcium influx (Nicholson and Kullmann, 2017). Moreover, LTP induced by AP trains showed two-way occlusion with the tetanus-induced LTP. LTP induced by either afferent tetanus or AP trains was associated with a decrease of the paired-pulse ratio and an increase of frequency of spontaneous EPSPs, suggesting involvement of presynaptic mechanisms in LTP expression. Interestingly, despite sharing mechanisms of induction and expression, tetanus-induced LTP was input-specific implying the need for presynaptic activation for the induction while LTP induced by AP trains was clearly independent of presynaptic activity at test synapses. One possible explanation for this difference is that nonsynaptic mechanisms of $[Ca^{2+}]_i$ rise, VGCCs, and release from internal stores were activated by the AP-only protocol sufficiently strong to produce calcium levels necessary for triggering plasticity all over the dendritic tree. During afferent tetanization, the $[Ca^{2+}]_i$ threshold for LTP was reached only around the activated synapses due to cooperative action of synaptic and nonsynaptic mechanisms of calcium rise, thus leading to input-specific LTP.

Inhibitory neurons from the visual cortex also express heterosynaptic plasticity. A protocol of intracellular tetanization: bursts of postsynaptic spikes induced by depolarizing pulses without presynaptic stimulation, which induced long-term plasticity in excitatory neurons from visual and auditory cortex (Volgushev et al., 1997, 2016; Lee et al., 2012), also induced plasticity in inhibitory neurons (Chistiakova et al., 2019). Intracellular tetanization could induce LTP or LTD or lead to no synaptic changes in neurons of both FS and non-FS types. LTP or LTD could be induced in unitary connections between simultaneously recorded pairs of neurons with controlled absence of presynaptic spikes during intracellular tetanization (**Figures 3A1–A3, B1–B3**) and at excitatory synapses activated with extracellular electric stimulation (**Figures 3C1–C3, D1–D3**). Because intracellular tetanization is a purely postsynaptic protocol applied without presynaptic stimulation, any plastic changes occurred at nonactivated synapses and, thus, were heterosynaptic. The direction and magnitude of heterosynaptic changes were correlated with the initial paired-pulse ratio, an index of release that is inversely related to release probability (Voronin, 1993; Dobrunz and Stevens, 1997; Murthy et al., 1997). This correlation was significant for all studied inputs pooled together (n = 233 inputs) as well as for the subpopulations of inputs to identified FS neurons and identified non-FS cells (**Figure 4**). Thus, heterosynaptic changes in inhibitory neurons were weight-dependent: Inputs with initially high paired-pulse ratio (low release probability, "weak" inputs) tended to be potentiated while inputs with initially low paired-pulse ratio (high release probability, "strong" synapses) tended to depress or did not change after intracellular tetanization. LTP and LTD were balanced in FS neurons: an average of changes over all inputs to FS neurons did not show significant difference from control. In non-FS neurons, a higher proportion of inputs expressed LTP than LTD (**Figure 4**), and an average of all inputs to non-FS neurons showed significant potentiation. This difference might be due to a combination of (i) the correlation of plasticity with initial paired-pulse ratio and (ii) significantly higher paired-pulse facilitation ratios in the inputs to non-FS vs. FS neurons, resulting in an increased probability of LTP in non-FS cells. Notably, heterosynaptic plasticity in inhibitory neurons could also be induced by a conventional STDP pairing protocol (Chistiakova et al., 2019). Pre-before-post pairing of synaptic stimulation with bursts of depolarization-evoked postsynaptic spikes induced LTP in 5 out of 10 paired inputs (**Figure 2**), LTD in two, and did not lead to changes in the remaining three inputs. On average, paired inputs were significantly potentiated. Plastic changes were

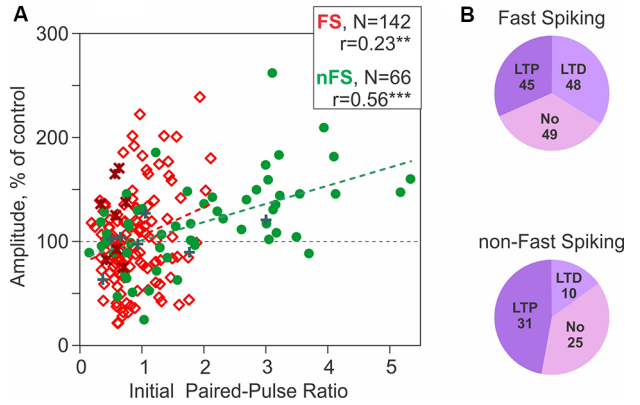


FIGURE 4 | Distinct paired-pulse ratio and heterosynaptic plasticity in FS and non-FS cells from visual cortex. **(A)** Changes of EPSP amplitude after intracellular tetanization plotted against initial paired-pulse ratio for inputs to FS (red diamond symbols, $n = 142$) and non-FS (green circles, $n = 66$) neurons. In each group, initial PPR and EPSP amplitude changes were significantly correlated (**$p < 0.01$ and ***$p < 0.001$). Data for unitary responses from paired recordings are shown as dark red asterisks (FS, $n = 8$) and dark green crosses (non-FS, $n = 8$). Note that for excitatory inputs to FS cells PPR < 2 was typical while in non-FS neurons PPR > 2 were frequently observed. **(B)** Pie charts showing frequency of occurrence of LTP, LTD, and no changes after intracellular tetanization in FS and non-FS cells. Number of inputs contributing to each group is shown within the charts (modified with permission from Chistiakova et al., 2019).

not restricted to the paired inputs: significant heterosynaptic LTP was observed in three, and LTD in 2 unpaired inputs out of 10. The average change in all unpaired inputs was not different from control. Thus, balanced heterosynaptic plasticity could be induced in inhibitory neurons by a conventional STDP protocol.

To summarize, there are form(s) of plasticity in interneurons that can be induced at synapses that were not active during the induction: heterosynaptic plasticity. Heterosynaptic plasticity can be induced by episodes of strong postsynaptic activity, evoked either purely postsynaptically by trains of depolarizing pulses (Nicholson and Kullmann, 2014, 2017; Chistiakova et al., 2019) or by conventional afferent tetanization (McMahon and Kauer, 1997; Cowan et al., 1998) or STDP pairing protocol (Chistiakova et al., 2019). Both LTP and LTD could be induced at heterosynaptic sites (Cowan et al., 1998; Chistiakova et al., 2019), whereby the direction of change is correlated with initial paired pulse ratio, suggesting weight-dependence of heterosynaptic plasticity (Chistiakova et al., 2019).

HOW DIVERSE FORMS OF PLASTICITY ACHIEVE HOMEOSTASIS OF EXCITATORY DRIVE OF INHIBITORY NEURONS

The majority of the input-specific plasticity discussed in this review can be classified as Hebbian-type associative plasticity. Associative plasticity is vital for adaptive fine-tuning of inhibitory systems to serve the multitude of their functions. However, Hebbian-type plasticity rules introduce an intrinsic positive feedback on synaptic weight changes, making synapses prone to runaway potentiation or depression and eventual saturation, and making neuronal activity prone to runaway activation or complete silencing. The need for homeostatic mechanisms to counteract these negative effects of Hebbian learning rules has been recognized since the earliest computational work on the subject (Von der Malsburg, 1973) and validated and specified in further work demonstrating that, to achieve both learning and stability of operation, neuronal networks need to be equipped with mechanisms of synaptic plasticity additional to Hebbain-type rules (e.g., Miller and MacKay, 1994; Miller, 1996; Oja, 1982; van Rossum et al., 2000; van Ooyen, 2001; Kempter et al., 2001; Wu and Yamaguchi, 2006; Morrison et al., 2008; Zenke et al., 2013). While these theoretical and computational studies had been focused on plasticity of excitatory connections between excitatory neurons, their results are not constrained by the transmitter identity of the output of the postsynaptic neuron. Inhibitory neurons driven by excitatory synapses equipped with Hebbian-type plasticity rules face the same problems: a tendency for runaway dynamics of synaptic weight changes and activity. Features of mechanism(s) counteracting these negative "side-effects" of Hebbian-type learning rules, established in theoretical work for excitatory neurons, are also relevant for mechanisms of homeostatic control of excitatory inputs to inhibitory neurons. The remarkable diversity of inhibitory neurons and plasticity mechanisms they express might impose additional constraints on the homeostatic mechanisms.

Required Features of Mechanisms Balancing Excitatory Drive of Inhibitory Neurons

Homeostasis of synaptic weights should operate at several levels, keeping cells and synapses in their respective operational range. At the level of the whole cell, one function of homeostatic mechanism(s) is to preserve an overall synaptic drive and avoid excessive input changes, which may lead to runaway activation or complete silencing of a neuron. In theoretical and model simulation studies, homeostasis of total synaptic drive is typically achieved by normalization: after each iteration of learning and changes of the weights at a subset of synapses, the weights of all synapses are adjusted so that their total sum (or squared sum) remains constant (Von der Malsburg, 1973; Oja, 1982). While details of the normalization procedure may affect specifics of learning abilities of model neurons and networks, the normalization effectively maintains synaptic drive of a cell at a certain level and prevents runaway dynamics of activity (e.g., Miller and MacKay, 1994; Kempter et al., 2001; van Ooyen, 2001; Elliott and Shadbolt, 2002; Wu and Yamaguchi, 2006). However, maintaining the total weight of all synapses does not prevent saturation of individual weights or elimination of individual synapses. Indeed learning in models with normalization typically leads to a bimodal distribution of synaptic weights with the weights of the "winner" synapses at the maximum and weights of other synapses close to zero (e.g., Song et al., 2000; van Rossum et al., 2000; Gütig et al., 2003; Morrison

et al., 2008). Synaptic weights of real neurons do not show such bimodal distributions, implying existence of additional mechanisms that prevent the saturation of weights of individual synapses. Thus, at the level of synapses, a function of homeostatic mechanisms is to prevent extreme changes of individual synaptic weights. This aspect of homeostasis is important for safeguarding synapses from elimination or saturation and keeping the weights in a range that allows for further learning and continued redistribution of weights to accommodate new memories (Volgushev et al., 2016).

One further general requirement for homeostatic mechanism(s) is the time scale of the induction of synaptic changes. Hebbian-type plasticity is induced within seconds or minutes, and to effectively counteract the tendency for runaway dynamics imposed by these fast plastic changes, homeostatic mechanism(s) should operate on a compatibly fast time scale (Wu and Yamaguchi, 2006; Zenke et al., 2013; Chistiakova et al., 2015; Zenke and Gerstner, 2017). Indeed, in most theoretical and simulation studies that use normalization to stabilize total synaptic drive, it is implemented directly into the equations for synaptic weight changes and, thus, operates on the exact same time scale as the associative plasticity (Von der Malsburg, 1973; Oja, 1982; Miller and MacKay, 1994; Miller, 1996). Research into the requirements for the time scale of homeostatic mechanisms showed that such mechanisms must induce "compensatory" plastic changes on the time scale that is same or similar to the time scale of Hebbian-type plasticity (Zenke et al., 2013; Zenke and Gerstner, 2017). One implication of this requirement is that mechanisms of "homeostatic synaptic scaling", which induce plastic changes after many hours or days of dramatic alterations of activity level (Watt and Desai, 2010; Wenner, 2011; Turrigiano, 2012; Keck et al., 2017) and play a role during development or recovery after injury and deafferentation, cannot serve the homeostatic function for fast-scale Hebbian-type plasticity (for further discussion see Chistiakova et al., 2014, 2015; Zenke and Gerstner, 2017).

A common requirement for both homeostatic regulation and fine-tuning of inhibitory systems by associative plasticity is that synaptic weights could be changed in both directions. Synaptic weights that can only change in one direction will progressively saturate, lose dynamic range, and have no ability to support further plasticity. Indeed, both LTP and LTD were observed in many excitatory connections to inhibitory neurons considered in this review. In some connections, however, plasticity in one direction prevails, e.g., only LTP was reported so far at synapses made by axon collaterals of CA1 pyramids onto str. pyramidale interneurons mediating feedback inhibition (Lamsa et al., 2007; Le Roux et al., 2013) while, almost exclusively, LTD was observed at mossy fiber inputs to CA3 str. lucidum interneurons (Maccaferri et al., 1998; Lei and McBain, 2002, 2004; Pelkey et al., 2005). Because most of these studies were aimed at in-depth analysis of specific forms of plasticity and experimental conditions were optimized accordingly, further research is needed to determine conditions for bidirectional plasticity at the diverse types of excitatory synapses to inhibitory neurons.

The vast number of plasticity rules present in interneurons, along with their heterogeneous electrophysiological properties and diverse patterns of activity, set two further important constraints for homeostatic mechanism(s). To be successful, homeostatic mechanism(s) must be generic enough *to respond to a wide range of plasticity rules and mechanisms* and robust enough to serve this function under *a wide range of activity patterns of inhibitory neurons*, expressing these diverse forms of homosynaptic plasticity.

The diversity of plasticity in interneurons demonstrates the need for a generic homeostatic mechanism but also highlights a point of convergence of the requirements for plasticity induction: the rise of $[Ca^{2+}]_i$. It has been argued, in the broad context of synaptic plasticity at excitatory synapses, that an emphasis on $[Ca^{2+}]_i$ rise as the triggering mechanism of plasticity can offer improved explanatory value over a fixation on learning rules, such as STDP (Lisman and Spruston, 2005, 2010). This philosophy might more accurately capture the relevant point of convergence for a variety of plasticity rules. In interneurons, nearly all forms of associative homosynaptic plasticity reported so far are calcium-dependent (with only one exception discussed above; Chen et al., 2009). A homeostatic mechanism that is triggered by intracellular calcium would fulfill the requirement of being generic. Heterosynaptic plasticity is a calcium-dependent phenomena, whether the source of $[Ca^{2+}]_i$ rise is strong local activation and local spread to inactive synapses or more global influx through voltage gated channels activated by back-propogating action potentials and amplified by release from internal stores. Importantly, this form of plasticity can be initiated by any event that causes strong activation of a neuron, firing, and a rise of $[Ca^{2+}]_i$ to a sufficiently high level, meaning that it is capable of being engaged by almost any activity pattern that induced any of the diverse forms of homosynaptic plasticity discussed above.

The requirement for the homeostatic mechanism to be robust means that it must successfully prevent runaway synaptic dynamics across a broad range of input patterns and postsynaptic firing of electrophysiologically heterogeneous inhibitory neurons equipped with diverse plasticity mechanisms.

To summarize, an ideal candidate mechanism for counteracting tendency for runaway dynamics imposed by Hebbian-type learning rules on weight changes of excitatory synapses and activity in interneurons should fulfill the following requirements. It should be able to prevent both runaway dynamics of the total excitatory drive as well as extreme changes at individual synapses and divergence of the weights of all synapses to either a maximum or zero. It should operate on the time scale that is compatible with the time scale of the mechanisms of associative plasticity. It should be able to change synaptic weights in both directions. It should be generic, i.e., could be induced in conjunction with any of the diverse forms of Hebbian-type plasticity expressed in interneurons, and robust, i.e., serve the homeostatic function under a wide range of inputs and firing patterns of inhibitory neurons equipped with diverse plasticity mechanisms. At the same time, the homeostatic mechanism should not prevent associative learning

and segregation of weights of synapses subject to different patterns of activity.

Weight-Dependent Heterosynaptic Plasticity as a Candidate Mechanism for Homeostatic Regulation of Excitatory Drive to Inhibitory Neurons

The following observed properties of heterosynaptic plasticity at excitatory inputs to inhibitory neurons in the visual cortex (Chistiakova et al., 2019) allow it to fulfill the above requirements and serve the function of homeostatic regulation of synaptic weight changes.

Results from our recent study show that, in the visual cortex, both major types of interneurons, FS and non-FS cells, express weight-dependent heterosynaptic plasticity (Chistiakova et al., 2019). Thus, this phenomenon might be a general and robust feature of neocortical inhibitory neurons, which express a broad range of specific mechanisms of associative plasticity discussed above (e.g. Lu et al., 2007; Sarihi et al., 2008; Huang et al., 2013). Weight-dependent heterosynaptic plasticity is also present in pyramidal neurons from visual and auditory cortex (Volgushev et al., 2000, 2016; Lee et al., 2012; Chen et al., 2013), extending its generality as a widespread feature of neurons. It is calcium-dependent in pyramidal neurons (Lee et al., 2012) and might be triggered by $[Ca^{2+}]_i$ rise in inhibitory neurons as well. In interneurons, heterosynaptic plasticity could be induced by the same episodes of postsynaptic activity (bursts of spikes) as associative plasticity but at nonactive synapses. Because bursts of spikes induce $[Ca^{2+}]_i$ rise in any type of interneuron tested so far (Goldberg and Yuste, 2005; Topolnik and Camiré, 2019; see section on calcium above), and calcium rise is the trigger for associative plasticity, heterosynaptic plasticity might share this fundamental requirement. Additionally, heterosynaptic changes could be induced by the same protocols as homosynaptic associative plasticity; hence, both forms of plasticity operate on the same time scale. Thus, heterosynaptic plasticity fulfills the requirement of being generic because it is triggered by the same activity patterns that induce associative plasticity; it is capable of playing the role of homeostatic regulator of synaptic changes in a broad variety of neuron types; and further, it operates on the same time scale of associative plasticity.

In weight-dependent heterosynaptic plasticity, the direction and magnitude of synaptic changes depend on the initial strength of the synapse. Synapses that are initially weak will have a disposition to potentiate while synapses that are initially strong will be predisposed for depression. This weight dependence sets a background constraint on synaptic weight changes, which is able to control unstable dynamics regardless of the specifics of activity patterns that tend to induce it. Indeed, computer model simulations demonstrate that weight-dependent heterosynaptic plasticity can robustly prevent runaway dynamics of synaptic weights and runaway activity of model neurons subject to widely different patterns of activity and equipped with widely different plasticity rules (Chen et al., 2013; Volgushev et al., 2016; Bannon et al., 2017). Such universal homeostatic "brakes" on runaway dynamics allow learning networks to benefit from a broad variety of plasticity rules, STDP windows, and activity patterns while, at the same time, robustly maintaining stable regime of operation and keeping excitatory synapses in operating range allowing for new learning (Chistiakova et al., 2015, 2019).

Because of its weight dependence, heterosynaptic plasticity has a normalizing effect on synaptic weights, which prevents both excessive increases and excessive decreases of weights. An increase of the weight of a synapse increases its predisposition for depression and vice versa; a decrease of the weight will increase predisposition of the synapse for heterosynaptic potentiation. As a result, synaptic weights are driven away from extreme values toward an equilibrium point within the operational range. Importantly, this effect of heterosynaptic plasticity is different from the effect of a formal mathematical normalization. Mathematical normalization preserves total synaptic drive to a cell but does not prevent runaway potentiation or depression of individual synapses. Learning in such models typically leads to distribution of synaptic weights around two modes, at the maximal weight and around zero (Song et al., 2000; van Rossum et al., 2000; Gütig et al., 2003; Morrison et al., 2008). In contrast, in models with weight-dependent heterosynaptic plasticity, learning does not lead to runaway potentiation or depression of individual synapses. Rather, the weights of all synapses in such models remain within the operation range (Chen et al., 2013; Volgushev et al., 2016; Bannon et al., 2017). Thus, weight-dependent heterosynaptic plasticity can robustly prevent both runaway dynamics of total synaptic drive and activity of a neuron as well as excessive changes of weights of individual synapses.

Importantly, weight-dependent heterosynaptic plasticity does not prevent segregation of weights of synapses subject to distinct patterns of input activity, e.g., groups of inputs with different frequency or correlation of presynaptic firing (Chen et al., 2013; Volgushev et al., 2016). Rather, this mechanism enhances segregation of synaptic weights by introducing a background force on synaptic weight changes. Associative plasticity drives weights of active synapses toward either maximal or minimal values. Heterosynaptic plasticity, triggered by the same episodes of strong activity that induce homosynaptic associative plasticity, drives synaptic weights of all synapses, including those inactive, away from the extremes. In this scenario, changes of active vs. inactive synapses are driven by contrasting forces and have different target weights (Chen et al., 2013; Chistiakova et al., 2014, 2015; Volgushev et al., 2016).

Therefore, we conclude that weight-dependent heterosynaptic plasticity represents a strong candidate mechanism for homeostatic regulation of synaptic weights and balancing their changes during ongoing associative synaptic plasticity and learning in inhibitory neurons.

Other Candidate Mechanisms for Balancing Plasticity at Excitatory Inputs to Inhibitory Neurons

Although the problem of balancing changes at excitatory synapses in interneurons during ongoing associative learning has

received little attention so far, a large body of research into the same problem in excitatory neurons has suggested a number of solutions that could be applicable for inhibitory neurons too.

Several solutions aim at balancing bidirectional homosynaptic changes. Indeed, balance of synaptic changes in neuron models can be achieved by careful adjustment of plasticity windows in depression-biased STDP rules (Song et al., 2000; Kempter et al., 2001; Gütig et al., 2003; Babadi and Abbott, 2010). Such models can learn, e.g., input pattern discrimination, by driving synaptic weights to either a maximum or zero while maintaining stable mean firing rates (e.g., Song et al., 2000; van Rossum et al., 2000; Gütig et al., 2003; Morrison et al., 2008). A problem with this solution is that it requires a precise correspondence between the amplitude and duration of potentiation and depression windows in STDP rules on the one hand and frequency and pattern of the input activity on the other. A change of input activity would destabilize the neuron. In a population of neurons with different STDP rules, a common activity pattern could be destabilizing for some neurons. For the heterogeneous population of cortical interneurons expressing broad range of plasticity rules as discussed in this review, such a solution is too constrained to be plausible.

An elegant solution allowing a dynamic adjustment of plasticity rules in a neuron is a sliding threshold for LTP and LTD as proposed in the Bienenstock-Cooper-Munro model (Bienenstock et al., 1982). One suggested mechanism here is dependence of intracellular calcium homeostasis on the recent history of synaptic changes and activity (Yeung et al., 2004). Mechanisms for activity-dependent regulation of calcium housekeeping are reported at least for some inhibitory neurons. Intense synaptic activity could change calcium signals evoked by back-propagating action potentials in dendrites of CA1 interneurons (Topolnik et al., 2009; Evstratova et al., 2011). However, plasticity in some types of interneurons may differ from excitatory cells in its dependence on $[Ca^{2+}]_i$ rises (e.g., Camiré and Topolnik, 2014) or tetanization frequency (Le Roux et al., 2013) and may follow cell type-specific STDP rules (Lu et al., 2007). Therefore, further research is needed to understand how a mechanism employing sliding thresholds for LTP and LTD may operate in interneurons. Theoretical and computational analysis is needed to understand how specific plasticity rules and calcium thresholds in interneurons should be regulated to reconcile associative learning with stability of neuronal operation, and the existence of corresponding mechanisms in diverse types of inhibitory neurons requires experimental validation.

One further mechanism that can reduce effects of the positive feedback of Hebbian-type rules on synaptic weight changes is weight-dependence of associative plasticity. This mechanism has been suggested theoretically (Oja, 1982), and experimental results in excitatory neurons show that, while weak synapses can express strong potentiation, stronger synapses potentiate less (Bi and Poo, 1998; van Rossum et al., 2000; Hardingham et al., 2007). Weight-dependence slows down saturation of synaptic weights and helps to achieve stable activity level of model neurons (van Rossum et al., 2000; Gütig et al., 2003). It is logical to assume that associative plasticity at excitatory synapses to inhibitory neurons is weight-dependent too; however, details of such dependence in diverse types of inhibitory neurons need to be explored.

One common drawback of the above mechanisms using bidirectional homosynaptic plasticity to balance synaptic changes is that they require presynaptic activation of a synapse to adjust its weight but cannot affect inactive synapses. This reliance on an external factor, input activity at a synapse, limits the ability of these mechanisms to serve as cell-intrinsic regulators of synaptic homeostasis. We conclude that, while solutions based on homosynaptic plasticity may help balance synaptic changes (see Chistiakova et al., 2014, 2015; for review and further discussion), these mechanisms are neither robust nor generic and cannot universally accommodate the vast range of activity patterns and learning rules observed in interneurons.

Mechanisms that employ heterosynaptic changes do not have these limitations. A broadly defined group of mechanisms related to competition for resources could affect both presynaptically active as well as inactive synapses and may help to maintain an overall balance of synaptic weights (Frey and Morris, 1997, 1998; van Ooyen, 2001; Elliott and Shadbolt, 2002; Fonseca et al., 2004). Mechanisms from this group may be involved in mediating the weight-dependent heterosynaptic plasticity considered above. An interesting mechanism of local balancing of synaptic changes has been described in inhibitory neurons from basolateral amygdala. In these neurons, potentiated or depressed synapses are surrounded by changes of the opposite sign producing a locally balanced profile of synaptic changes (Royer and Paré, 2003). While neither competition for resources nor local balancing were studied in cortical inhibitory neurons so far, both mechanisms have potential to mediate a robust homeostatic regulation of excitatory inputs to cortical interneurons.

Finally, nonsynaptic mechanisms regulating intrinsic excitability could accompany synaptic plasticity in excitatory neurons (Bliss and Lomo, 1973; Daoudal et al., 2002; Zhang and Linden, 2003; Frick et al., 2004; Karmarkar and Buonomano, 2006; Fink and O'Dell, 2009; Sehgal et al., 2013). A whole neuron or just an activated dendritic branch may change excitability, thus affecting the constituent synapses. The effect of excitability changes may be either homeostatic, counteracting synaptic changes (Zhang and Linden, 2003; Karmarkar and Buonomano, 2006), or anti-homeostatic, enhancing synaptic changes (Frick et al., 2004; Fink and O'Dell, 2009). Note that the original study of Taube and Schwartzkroin (1987) did not find excitability changes in CA1 interneurons after tetanic stimulation. However, this issue requires further studies in other types of cortical interneurons, which express a remarkable heterogeneity of electrophysiological properties.

To summarize, we conclude that, among the mechanisms for homeostatic regulation of excitatory inputs to inhibitory neurons, considered above, weight-dependent heterosynaptic plasticity represents a strong candidate. It is a generic and robust mechanism that could serve the function of overall constraint of total synaptic weight (preventing extreme changes of synaptic drive and runaway activity) as well as the function of keeping weights of individual synapses in working range. Regardless, it

is unlikely to be the only mechanism at work and additional mechanisms operating at the synaptic, cellular, and network levels might be involved in homeostatic regulation of activation of inhibitory neurons.

OUTLOOK AND OPEN QUESTIONS: WHY IS PLASTICITY IN INTERNEURONS INTERESTING?

Inhibitory interneurons exhibit unique morphology, electrophysiology, and patterns of protein expression, which clearly differentiate them from excitatory cells but, at the same time, are highly heterogeneous among themselves. This remarkable diversity of inhibitory neurons opens up an opportunity to address both cell-type and connection-specificity of plasticity rules as well as to distill basic rules common for all plastic synapses.

Diversity of distinct roles played by specific types of inhibitory interneurons in neuronal networks allows us to ask whether there are specific rules and mechanisms of plasticity that help to refine that circuit function. At the level of microcircuits, this could be studied, e.g., by comparison of plasticity in feed-forward vs. feedback inhibitory systems, or plasticity in inhibitory neurons targeting the dendrites and, thus, shaping input integration in pyramidal neurons vs. interneurons targeting the axon and the soma of pyramidal neurons and, thus, controlling their output. At the level of larger-scale cortical networks, relevant comparison(s) could be between plasticity in groups of inhibitory neurons serving distinct functions, e.g., mediating feature selectivity, shaping temporal patterns of activity and rhythms, or controlling and restricting spatial spread of activity. Progress of research that defines specific subpopulations and types of inhibitory neurons serving these and other specific functions opens up opportunities to address these kinds of questions.

Finally, achieving a better understanding of plasticity in inhibitory neurons has intrinsic value for the field of plasticity as a whole. One common motif of plasticity of excitatory inputs to inhibitory neurons discussed in this review is that individual synapses are typically equipped with mechanisms, such as distinct sources of $[Ca^{2+}]_i$ rise and intracellular machinery, which can support multiple forms of plasticity. How do these diverse mechanisms and forms of plasticity interact at one synapse? Most of the research has been aimed at disentangling the effects of specific mechanisms while their interaction received little attention so far. A further step toward understanding synaptic plasticity in inhibitory neurons would require knowledge of forms and mechanisms of plasticity that can be induced by natural patterns of activity, typical for each specific type of inhibitory neurons. The ultimate answer to this question would require studies during natural activity *in vivo* and should include modulation of plasticity rules by natural brain states.

AUTHOR CONTRIBUTIONS

NB, MC and MV designed and wrote the manuscript.

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REVIEW
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TNF-Mediated Homeostatic Synaptic Plasticity: From *in vitro* to *in vivo* Models

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Since it was first described almost 30 years ago, homeostatic synaptic plasticity (HSP) has been hypothesized to play a key role in maintaining neuronal circuit function in both developing and adult animals. While well characterized *in vitro*, determining the *in vivo* roles of this form of plasticity remains challenging. Since the discovery that the pro-inflammatory cytokine tumor necrosis factor-α (TNF-α) mediates some forms of HSP, it has been possible to probe some of the *in vivo* contribution of TNF-mediated HSP. Work from our lab and others has found roles for TNF-HSP in a variety of functions, including the developmental plasticity of sensory systems, models of drug addiction, and the response to psychiatric drugs.

Keywords: inflammation, homeostatic plasticity, addiction, developmental plasticity, TNF

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HOMEOSTATIC SYNAPTIC PLASTICITY (HSP)

The maintenance of neural circuit function is a dynamic balance of several different types of synaptic plasticity. Synaptic strength can be modified by two broad types of plasticity mechanisms: Hebbian and non-Hebbian. Long term potentiation (LTP) and long term depression (LTD) are examples of Hebbian plasticity, where the strength of a given synapse is adjusted in response to synchronous activity (Malinow and Malenka, 2002). It is proposed as a mechanism of information storage and is thought to underlie the processes of learning and memory. On the other hand, non-Hebbian plasticity is posited to serve a homeostatic role, maintaining the stability of neural circuits in the face of changing conditions (Turrigiano et al., 1998).

Homeostatic synaptic plasticity (HSP) serves to keep neuronal activity levels in a range optimal for neurotransmission. It was first described as a response to prolonged perturbations in overall activity levels: when firing rates decrease, it serves to augment excitatory synaptic strength to normalize activity (sometimes referred to as upscaling), and when firing rates increase, the opposite occurs (downscaling). This phenomenon has been described in a variety of systems, including the mammalian central nervous system and the *Drosophila* neuromuscular junction (NMJ). The HSP at the *Drosophila* NMJ appears mechanistically distinct from HSP in the mammalian CNS, and therefore will not be covered here (for reviews of this topic see Davis and Müller, 2015; Frank et al., 2020). It should be noted, however, that both innate immune molecules and glia have recently been implicated in HSP at the *Drosophila* NMJ (Harris et al., 2015; Wang et al., 2020).

For the mammalian system, since being first described in the late nineties (O'Brien et al., 1998; Turrigiano et al., 1998), a great diversity of molecules have been implicated in homeostatic alterations in synaptic strength. These include proteins involved in calcium signaling (Thiagarajan et al., 2002; Ibata et al., 2008), transmembrane signaling proteins including MHCI and integrins (Goddard et al., 2007; Cingolani et al., 2008), endocytic proteins like Arc (Rial Verde et al., 2006; Shepherd et al., 2006), cytoskeletal proteins such as synaptopodin (Vlachos et al., 2013) and Homer1a (Hu et al., 2010), receptor-interacting proteins including PICK1 (Anggono et al., 2011), Narp (Chang et al., 2010), polo-like kinase 2 (PLK2; Seeburg et al., 2005), and dystroglycan (Pribiag et al., 2014), and secreted factors including BDNF (Rutherford et al., 1998), retinoic acid (Aoto et al., 2008; Chen and Napoli, 2008), and the pro-inflammatory cytokine tumor necrosis factor (TNF; Stellwagen and Malenka, 2006).

From these reports, it is clear that HSP is more diverse than originally described; it is not a single process, but rather many mechanisms operating either in conjunction or in parallel, responding to distinct circumstances. For example, there is evidence for cell-specific forms of HSP distinct from HSP induced by global activity suppression (Burrone et al., 2002). On a subcellular level, there are reports of homeostatic control of local dendritic regions and synapse-specific forms of HSP (Sutton et al., 2006; Kim and Tsien, 2008; Beique et al., 2011; Petrus et al., 2015; Barnes et al., 2017). Furthermore, multiple types of HSP have emerged operating at different spatial and temporal scales (Lee et al., 2014), and even the global form of HSP may still have distinct temporal components, with a more rapid retinoic acid-dependent form (Chen et al., 2014) and a slower, longer-lasting TNF-dependent form (Stellwagen and Malenka, 2006; Steinmetz and Turrigiano, 2010). It is important to note that the TNF-dependent and retinoic acid-dependent mechanisms of HSP only mediate upscaling, while a similarly varied but distinct set of molecules and mechanisms contribute to downscaling (Seeburg et al., 2008; Pribiag et al., 2014). Thus upscaling and downscaling are likely to be separate phenomena.

In addition to assuming HSP would have a single mechanism, early work also suggested that these changes occur in a multiplicative fashion: synaptic strength is adjusted by the same factor such that the relative differences in synapses are preserved (Turrigiano et al., 1998) and therefore the information stored in the synaptic weight difference would also be preserved (Turrigiano and Nelson, 2004). As a result, HSP is often referred to as synaptic scaling. This hypothesis may not strictly hold: recent reports that while the multiplicative nature of scaling holds true on a population level, there is variable scaling at the level of individual synapses (Wang et al., 2019; Hanes et al., 2020). Recent results have also challenged the notion that changes in cell firing are the driver for HSP, as maintaining spiking while blocking synaptic function still leads to HSP (Fong et al., 2015). Consequently, we shall avoid the term synaptic scaling, and only use HSP instead.

One of the first proteins placed within this pathway was TNF (Stellwagen and Malenka, 2006). This review article will first explore the effect of TNF on synapses, and then explore the models and systems in which TNF mediates different forms of homeostatic plasticity.

TNF IN THE BRAIN

Historically, the central nervous system (CNS) was considered a site that was kept separate from the peripheral immune system, with immune signaling molecules excluded from the CNS by the blood-brain barrier (BBB; Barker and Billingham, 1977). The two systems were thought as so distinct that a specific term was coined to describe how they were kept separate: immune privilege. The lack of conventional lymphatic vessels as well as the extended survival of foreign tissue grafts in the brain suggested that the CNS is not capable of the same immune responses that are present in the periphery. The first evidence to the contrary was the discovery that under some pathological conditions, cytokines, mediators of immune responses, are produced in the brain (Hopkins and Rothwell, 1995). Furthermore, it is now becoming evident that immune privilege is far from absolute (Galea et al., 2007) and immune molecules are present in the nervous system even under non-pathological conditions and play a role in regulating synaptic function (Vitkovic et al., 2000). In particular, the pro-inflammatory cytokine TNF regulates synaptic properties and has been ascribed a role in HSP (Stellwagen and Malenka, 2006).

TNF AND TNF RECEPTOR OVERVIEW

Cytokines are small protein molecules released by cells that serve as messengers between immune cells, modulating their interactions and behavior. TNF is one such pleiotropic cytokine that has many well-characterized roles including mediating inflammatory responses, cell differentiation, and organogenesis (Locksley et al., 2001; Santello and Volterra, 2012). It is transcribed as a single pass transmembrane pro-protein which can signal directly in its membrane-bound form (Grell et al., 1995). It can also be cleaved by the matrix metalloprotease ADAM17 (otherwise known as TNF- converting enzyme; TACE) to release soluble TNF (Kriegler et al., 1988; Black et al., 1997). Regardless of its cleavage status, TNF forms trimers which are the active form, responsible for signaling at TNF receptors (TNFRs; Smith and Baglioni, 1987).

TNF is produced in the CNS during a variety of inflammatory pathologies. It is upregulated after exposure to bacterial and viral proteins (Lokensgard et al., 2001; Kielian et al., 2002), but can also be induced by intrinsically-derived CNS insults. It is increased in diseases such as multiple sclerosis (MS; Hofman et al., 1989), Alzheimer's disease (AD; Fillit et al., 1991), Parkinson's disease (PD; Mogi et al., 1994), among others. Acute injuries such as CNS trauma also result in TNF expression (Ross et al., 1994). In addition to a role in the CNS in response to these various pathologies, both TNF mRNA and protein can be found in the non-inflamed brain (Vitkovic et al., 2000), suggesting functions even under non-pathological conditions.

The concentration of TNF is likely significant—low, physiological levels seem to modulate neuronal function, considerably below the high concentrations found in

inflammatory or disease states. TNF levels are constitutively low and only modestly increase (3–5 fold) with activity blockade or other manipulations (Stellwagen and Malenka, 2006; Lewitus et al., 2016). This review article will address TNF at physiological, not pathological, concentrations.

TNF can signal through two receptors—TNFR1 and TNFR2—which differ in their expression pattern, binding affinity for the different forms of TNF, and their downstream signaling pathways (MacEwan, 2002). TNFR1 can efficiently bind both soluble and membrane-bound TNF while TNFR2 has a much higher affinity for binding to membrane-bound TNF (Grell et al., 1995). TNFR1 is constitutively expressed by cells in the CNS (Kinouchi et al., 1991) and periphery (Aggarwal, 2003), whereas expression of TNFR2 is more limited, with reports mainly in endothelial and immune cells (Aggarwal, 2003) as well as reports of expressions in some neurons (Neumann et al., 2002). TNFR1 signaling is complex, and can result in proliferation, activation, and apoptosis, depending on the context, while TNFR2 signaling is generally anti-inflammatory and pro-survival (Wajant et al., 2003). Additionally, membrane-bound TNF can signal in the reverse direction when complexed with TNFR1 in both the immune and nervous systems (Harashima et al., 2001; Kisiswa et al., 2013).

TNF EFFECTS ON PYRAMIDAL NEURONS

TNF is capable of modulating both presynaptic and postsynaptic function in neurons (**Figure 1A** and **Table 1**). A key measure of the presynaptic function is the frequency of miniature postsynaptic currents, which are the post-synaptic response to the unitary release of neurotransmitters. The frequency of these currents is generally taken to be a reflection of the probability of release of transmitter from the presynapse, as well as the number of synapses on the cell, while changes in amplitude are generally assumed to be due to post-synaptic changes. It should be noted that there are several ways these assumptions can fail, but they hold true for most situations. Treatment of cultured hippocampal neurons with TNF increases miniature excitatory postsynaptic current (mEPSC) frequency in pyramidal neurons (Grassi et al., 1994; Beattie et al., 2002). This effect was observed during direct, short term application of TNF to individual neurons, but is more difficult to detect with longer-term treatments and cross-cell comparisons (e.g., Stellwagen et al., 2005; Stellwagen and Malenka, 2006). Whether the increase in release probability is temporary or whether it is lost in the noisiness of cross-cell comparisons is uncertain. One report suggests that the effect on release probability may not be direct, but rather through a mechanism involving the glial release of other factors such as ATP (Santello et al., 2011).

The modulatory effects of TNF are not unique to excitatory synapses—miniature inhibitory synaptic current (mIPSC) frequency decreases with TNF treatment of hippocampal cultures (Pribiag and Stellwagen, 2013). Furthermore, the application of a soluble version of TNFR1, which serves to block TNF signaling by acting as a TNF sink, results in a decrease in the baseline mEPSC frequency, suggesting that ongoing TNF signaling is required to maintain normal synaptic function. This indicates that not only is TNF capable of modulating synaptic function in response to its administration, but also that its constitutive secretion is responsible for maintaining synapses in their baseline state. Taken together, these effects are all consistent with an overall outcome of increased synaptic transmission in the presence of TNF, suggesting an important role of TNF under non-pathological, non-inflammatory conditions in the CNS.

The most well-established mechanism by which TNF modulates synapses is through the post-synaptic trafficking of neurotransmitter receptors. Excitatory neurotransmission is mainly accomplished through the activation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-type glutamate receptors (AMPARs), and their abundance at the synapse largely determines the neuronal response to a given stimulus. They are therefore a frequent point of regulation for the expression of synaptic plasticity (Malinow and Malenka, 2002).

Early studies focused on the effects of exogenous TNF administration on mature cultured hippocampal and cortical neurons. Treatment of dissociated hippocampal cultures with TNF results in a rapid (within 10–15 min) and large-scale trafficking of AMPARs (doubling) to the surface of pyramidal neurons, as determined by immunocytochemistry (Beattie et al., 2002; Ogoshi et al., 2005; Stellwagen et al., 2005). These newly-inserted receptors colocalize with synaptic markers, indicating that they can contribute to synaptic function (Beattie et al., 2002). It is also important to note the potential role of TNF in setting basal AMPAR levels. Application of a soluble version of TNFR1 resulted in the reduction of surface AMPAR staining to below baseline (Beattie et al., 2002), again suggesting that TNF is important for continual maintenance of synaptic components. Also, cultured cortical neurons prepared from TNFR1 knockout animals have fewer surface GluA1 clusters (He et al., 2012), confirming a role for TNF in maintaining AMPAR levels.

The synaptic effects of TNF were also more directly tested by electrophysiology on both cultured neurons and the more intact preparation of acute hippocampal slices. The amplitude of miniature postsynaptic currents are the neuronal response to the unitary release of neurotransmitters, and as such, it is taken to be reflective of the receptor content of the postsynaptic cell. Administration of TNF to both dissociated neuronal cultures and acute slices resulted in an increase in mEPSC amplitude on pyramidal neurons (Stellwagen et al., 2005), which is consistent with immunocytochemistry data indicating that TNF strengthens synapses. It is interesting to note that longer-term exposure to TNF can lead to different results—24 h treatment led to a modest decrease in whole-cell AMPA-induced currents (Furukawa and Mattson, 1998), indicating that time course may play a role in the biological outcome of TNF exposure.

It is also important to consider the subunit composition of AMPARs, as it is critical to their biological function. AMPARs are assembled as tetramers of the GluA1–GluA4 subunits (Wisden and Seeburg, 1993). In general, they are found as heteromers of either GluA1 and GluA2, or heteromers of GluA2 and GluA3, but can be found as GluA1 homomers (Wenthold et al., 1996; Shi et al., 2001). The presence of

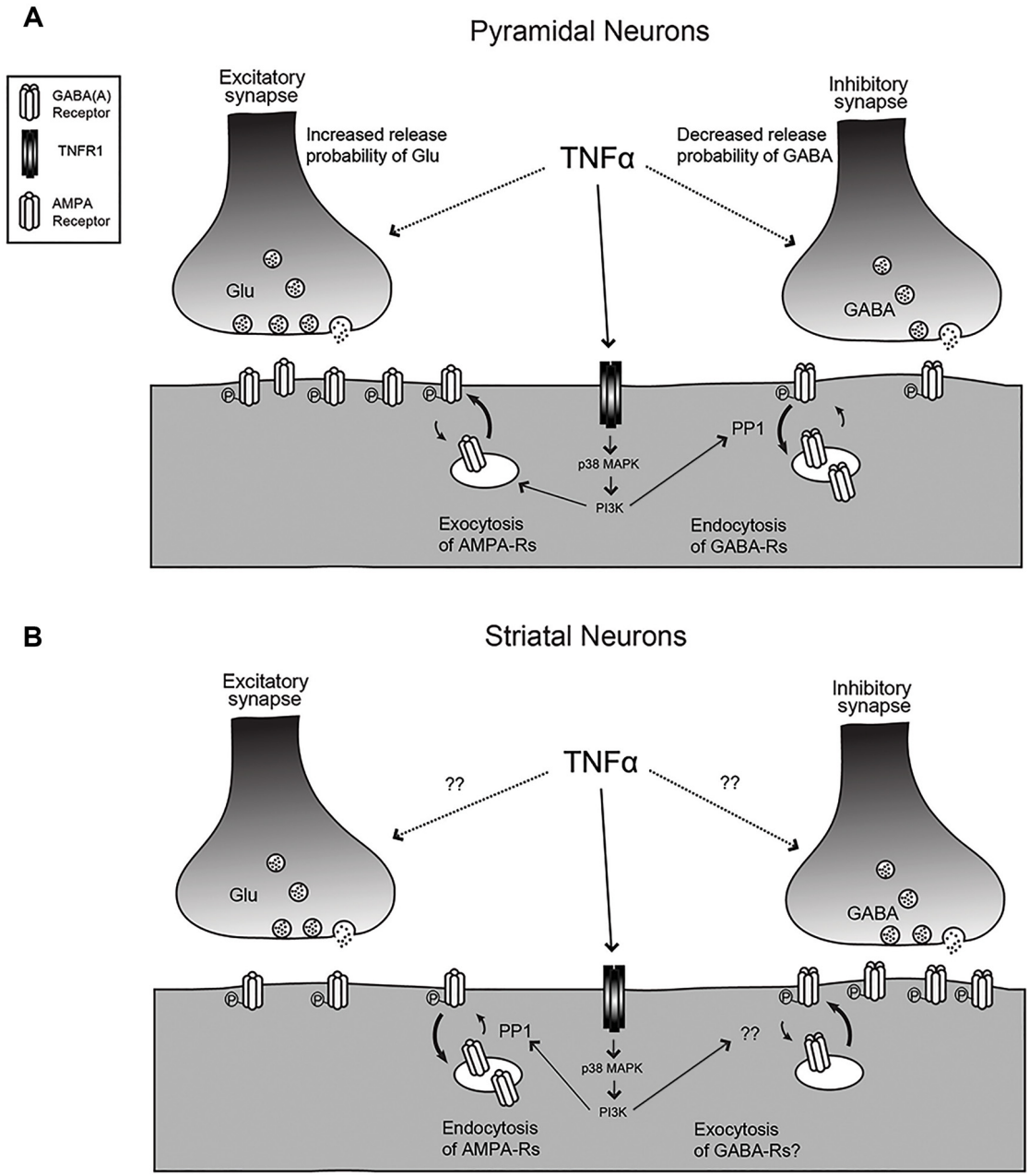

FIGURE 1 | The effects of tumor necrosis factor (TNF) on synaptic function. **(A)** For hippocampal or cortical pyramidal neurons, TNF treatment leads to an increase in release probability and an increase in AMPA receptor content at excitatory synapses but a decrease in release probability and decrease in $GABA_A$ receptor content at inhibitory synapses. The mechanisms for the change in release probability are unknown but the post-synaptic receptor trafficking requires p38-MAP kinase and PI3 kinase and the receptor endocytosis is dependent on protein phosphatase 1 (PP1). **(B)** The response is reversed for medium spiny neurons (MSNs) in the striatum and neurons in the habenula. Here, TNF causes endocytosis of AMPA receptors and may cause exocytosis of GABA receptors. Changes in release probability have not been documented. Figure adapted from Pribiag and Stellwagen (2013).

GluA2 in receptor complexes is critical: it is the subunit that confers calcium impermeability to the AMPAR complex (Burnashev et al., 1992). The biological consequences of calcium permeability are wide-reaching due to the importance of calcium to many synaptic processes. It is critical to multiple forms of plasticity (Zucker, 1999), and is part of the cascade of excitotoxic cell death which is characteristic of numerous neurological pathologies (Choi, 1992; Dong et al., 2009). It is then particularly intriguing that several groups have reported that the AMPARs trafficked to the cell surface in response to TNF treatment are

TABLE 1 | Details of the effects of tumor necrosis factor (TNF) on synaptic function.

Preparation	TNFα treatment	Result	Reference
Rat hippocampal cultures	50–180 ng/ml, 2–5 min	↑ Glutamate release probability	Grassi et al. (1994)
	10–1,000 ng/ml, 15 min	↑ Glutamate release probability ↑ Surface AMPARs	Beattie et al. (2002)
	50–250 ng/ml, 45 min	↓ GABA release probability ↓ Surface GABARs ↓ GABAR current	Pribiag and Stellwagen (2013)
	100 ng/ml, 15–20 min	↑ Surface AMPARs ↑ AMPAR current ↓ Surface GABARs ↓ GABAR current	Stellwagen et al. (2005)
Mouse acute hippocampal slices	100 ng/ml, 15 min	↑ Surface AMPARs	Ogoshi et al. (2005)
Rat acute hippocampal slices	1,000 ng/ml, 2–3 h 100 ng/ml, 1–2 h	↑ AMPAR current ↓ GABAR current	Stellwagen et al. (2005) and Lewitus et al. (2014)
Rat acute striatal slices	100 ng/ml, 1–2 h	↓ AMPA/NMDA ratio ↓ Surface AMPARs	Lewitus et al. (2014, 2016)
Rat acute lateral habenula slices	100 ng/ml, 1 h	↓ AMPA/NMDA ratio	Valentinova et al. (2019)

permeable to calcium (Ogoshi et al., 2005; Stellwagen et al., 2005) because of the potential implications for neurological disease, which often involve neuroinflammation. It has also been reported that after initial, rapid exocytosis of GluA2-lacking receptors within minutes, AMPARs are slowly switched to GluA2-containing surface receptors (Leonoudakis et al., 2008) with longer treatment, suggesting that the outcome of TNF application is dependent on the time course of application, and responses may occur in more than one phase.

TNF can also modulate inhibitory neurotransmitter receptors. γ-aminobutyric acid receptors (GABARs) are the main mediators of fast inhibitory transmission in the brain (Jacob et al., 2008) and are critical to the dynamics of neural circuits. An early study in hippocampal culture and acute hippocampal slices shows that TNF treatment leads to both a decrease in surface GABAR staining, as well as a decrease in mIPSC amplitude, consistent with an overall decrease in inhibitory neurotransmission (Stellwagen et al., 2005). A subsequent report determined that the mechanism of TNF-induced GABAR regulation is through p38 MAPK, PI3K, and protein phosphatase 1 (PP1), leading to the dephosphorylation of the GABARs and their endocytosis from the cell surface (Pribiag and Stellwagen, 2013). Taken together, the overall effect of TNF-induced receptor trafficking—increased surface AMPARs and decreased surface GABARs—is to increase the strength of synapses. Because exogenous administration is capable of rapidly modulating both excitatory and inhibitory synapses, TNF emerges as a potentially critical regulator of circuit excitability.

TNF EFFECTS ON STRIATAL NEURONS

In addition to this detailed work on the effects of TNF on the glutamatergic neurons of the hippocampus and cortex, its function has also been characterized on the inhibitory medium spiny neurons (MSNs) of the striatum (**Figure 1B**). In experiments where acute striatal slices were treated with TNF, there was a decrease in excitatory synaptic strength in corticostriatal synapses as measured by electrophysiology, as well as a decrease in surface AMPARs measured biochemically (Lewitus et al., 2014). These changes are more prominent on the direct pathway MSNs than on indirect pathway neurons (Lewitus et al., 2016). It is intriguing that in this context, the AMPARs that are trafficked are GluA2-lacking receptors, the same subtype that is trafficked in response to TNF in the hippocampus, although in the opposite direction. While this initially appears contradictory, the result of a decrease in excitatory synaptic strength in the striatum is a decrease in its inhibitory output through MSNs. Therefore, the overall effect of TNF administration is increasing the strength of neural circuits, which is consistent with the overall effect in the hippocampus and cortex.

HSP IN DISSOCIATED CULTURE

The exogenous application of TNF has clear effects on synapses, so it is, critical to consider the biological conditions that lead to TNF release in the CNS. Examining the role of TNF in various forms of synaptic plasticity, therefore, gives context to the effects on neurotransmitter receptor trafficking observed by TNF administration.

TNF is critical to the process of scaling up excitatory synaptic strength in response to prolonged activity blockade (Stellwagen and Malenka, 2006). Depriving dissociated hippocampal cultures of activity for 48 h using tetrodotoxin (TTX) to prevent action potential generation by blocking sodium channels results in an increase in surface AMPARs and a decrease in surface GABARs. This modulation of surface receptors gives rise to the expected electrophysiological changes: mEPSC amplitude increases, while mIPSC amplitude decreases, giving an overall increase in synaptic strength. Synaptic changes are accompanied by an increase in TNF in the cell culture medium, suggesting that it could be involved in the response to activity deprivation. This is supported by experiments showing that treatment of cultures with a soluble TNFR1, which blocks TNF signaling, during activity blockade prevents the upscaling of synaptic

strength. Furthermore, TNF KO animals lack HSP in response to activity deprivation, supporting its involvement in synaptic strengthening. Altogether, this is clear evidence that TNF is required for synaptic upscaling. It is important to note, however, that TNF does not appear to be required for the downscaling of synapses in response to activity elevation (Stellwagen and Malenka, 2006).

Interestingly, there is a report suggesting that the TNF requirement in HSP is time-dependent (Steinmetz and Turrigiano, 2010). TNF may be dispensable for early (<6 h) stages of HSP, but that its prolonged blockade with a soluble TNFR1 does prevent late (24 h) stages of HSP, which is not necessarily inconsistent with previous reports characterizing TNF involvement in the response to 48 h of activity blockade. Rather, it implies that there are two stages to the process of scaling up synapses. Further experimentation will be required to determine the distinctions between early and late phase HSP as it relates to TNF.

The precise subunit composition of the AMPARs trafficked during HSP has not been completely characterized. However, increases in surface GluA1 staining were observed following activity deprivation (Stellwagen and Malenka, 2006). Together with previous evidence in the same culture system showing that TNF treatment resulted in exocytosis of GluA2-lacking AMPARs (Stellwagen et al., 2005), it seems likely that the same type of AMPARs would be trafficked in this form of HSP. Furthermore, other reports of TTX-induced homeostatic plasticity are generally supportive of this, showing increased levels of GluA2-lacking AMPARs after activity deprivation (Thiagarajan et al., 2005; Sutton et al., 2006; Aoto et al., 2008; Hou et al., 2008). Additionally, there is a report suggesting that phosphorylation of GluA1 is required for synaptic scaling (Kim and Ziff, 2014). Some reports show that GluA2 is required for TTX-induced scaling using GluA2 knockdown cortical cultures (Gainey et al., 2009) and organotypic hippocampal slice cultures (Ancona Esselmann et al., 2017). On the other hand, a study was also performed using knockout cultures for GluA1, GluA2, and GluA3 indicating that there is no subunit requirement for TTX-induced upscaling (Altimimi and Stellwagen, 2013), perhaps as a result of compensation by alternate compositions of AMPARs in the absence of a given subunit.

Understanding the source of TNF during HSP gives valuable insight into the mechanics of the process. Early studies in culture indicated that glia produce TNF basally, and that conditioned media from glial cultures was able to induce exocytosis of AMPARs neurons (Beattie et al., 2002), but it was not clear whether this was the mechanism at play during HSP. Using Banker cultures to plate neurons onto a feeder layer of glia that is physically separate, a genetic approach allowed for precise characterization of the roles of individual cell types in TNF secretion. Wild type neurons cultured with TNF KO glia were unable to express HPS whereas TNF KO neurons cultured with wild type glia behaved similarly to controls suggesting that glial TNF mediates HSP (Stellwagen and Malenka, 2006).

While implicating glia, this work did not identify the subtype involved. Within the central nervous system, TNF is largely produced by glia, including both astrocytes and microglia. TNF is occasionally seen (both at the RNA and protein level) in neurons, but typically only in pathological conditions. Which cells secrete the low level of TNF regulating HSP is currently unclear. *In vivo*, varying manipulations result in TNF production from astrocytes (Duseja et al., 2015) and microglia (Lewitus et al., 2016). During HSP, astrocytes are the best positioned to monitor the activity of synapses and are the likely source of HSP-mediating TNF, but this remains to be determined.

For many years, glia were assumed to merely provide physical and trophic support for neuronal function. The finding that glial TNF is required for HSP lends weight to the more recent observation that glia are capable of being active players at the synapse, shaping properties of neurotransmission through the secretion of modulatory factors.

HSP IN ENTORHINO-HIPPOCAMPAL SLICE CULTURES

While dissociated culture is a valuable tool for the dissection of neural function, it is important to verify the biological relevance of the information gleaned from them. As such, performing experiments in more intact systems is necessary to examine whether findings in dissociated culture hold true outside of that system. In that vein, HSP has been studied in entorhino-hippocampal slice cultures.

The entorhinal cortex is the major input and output structure for the hippocampus and is often thought of as a gateway between the hippocampus and cortex. Projections from the entorhinal cortex to the dentate gyrus termed the perforant path, have been studied extensively in terms of both structure and plasticity for many years (Bliss and Lomo, 1973; Douglas and Goddard, 1975; Witter, 2007). These connections can be preserved in a slice culture system, allowing for the examination of a physiologically relevant neural circuit in the context of HSP.

Entorhino-hippocampal slice cultures also allow for a more physiological activity manipulation than bath application of TTX. Entorhinal denervation by lesioning the inputs to the dentate provides a paradigm in which synapses in the dentate can be studied in terms of their response to a decrease in excitatory input. When this type of lesion is performed, it results in a homeostatic increase in mEPSC amplitudes in the dentate which reaches its maximum 3–4 days post-lesion (Vlachos et al., 2012, 2013), similar to the effects of TTX treatment on dissociated cultures. Further addition of TTX to denervated slice cultures did not lead to an additional increase in synaptic strength, suggesting that a common pathway underlies the response to both manipulations.

TNF was also required for this form of HSP. Slice cultures either made from TNF KO animals, or slice cultures treated with soluble TNFR to block signaling lacked the late-stage synaptic strengthening at 3–4 days post-lesion (Becker et al., 2013). Furthermore, TNF is likely secreted by glia in this context, similar to early HSP experiments. Using *in situ* hybridization in concert with immunofluorescent labeling of astrocytes, the authors show an increase in TNF mRNA in astrocytes after denervation. Though this does not exclude a contribution of TNF

from other cell types, it does suggest that astrocytes are capable of supplying TNF during denervation-induced HSP, though further experimentation is necessary to ascertain whether astrocytic TNF is a requirement.

It is, however, important to note that not all components of HSP are recapitulated in the slice culture denervation model. Recently, a study showed that while increased mEPSC amplitude in dentate granule cells is observed in slice culture, there is no concomitant decrease in mIPSC amplitude (Lenz et al., 2019) that is characteristic of HSP in dissociated neuron-glial cultures (Turrigiano et al., 1998; Kilman et al., 2002; Stellwagen and Malenka, 2006). The use of *in vitro* models has utility due to the ease of performing manipulations, but must be validated *in vivo*.

TNF IN META-PLASTICITY

There is also evidence that TNF is involved in forms of plasticity other than HSP. While TNF regulates AMPA receptor trafficking, it is not required for either LTP or LTD (Stellwagen and Malenka, 2006). However, TNF may be capable of altering the threshold of induction for these Hebbian forms of plasticity in a process called meta-plasticity. Prior TNF treatment can inhibit or reduce subsequent hippocampal LTP in various circumstances (Tancredi et al., 1992; Cunningham et al., 1996; Butler et al., 2004; Pickering et al., 2005), often at lower doses and shorter applications than for TNF-mediated receptor trafficking. Recent work has clarified these findings, determining that TNF is capable of inducing meta-plasticity (Hulme et al., 2012; Singh et al., 2019), where prior activity reduces the induction of LTP but increases the induction of LTD (Hulme et al., 2012). The mechanism for this meta-plastic shift is uncertain but may be distinct from HSP. The relationship between meta-plasticity and HSP is also currently unclear—both can provide stability to neural networks, and may represent aspects of a larger, integrative negative feedback system. Importantly, many of the *in vivo* functions of TNF discussed below induce synaptic changes that rely on sustained TNF signaling, and so are more likely due to its role in HSP rather than meta-plasticity. But further work will need to clarify the situation for any particular *in vivo* function for TNF.

MONOCULAR DEPRIVATION-INDUCED PLASTICITY

The visual system has also offered insight into the role of TNF-dependent plasticity in intact animals. During early development, the visual system is highly plastic at a time referred to as the critical period (Hubel and Wiesel, 1970). If one eye is deprived of input by suturing it shut—an experimental paradigm termed monocular deprivation (MD)—several stages of plasticity are engaged in the binocular zone of the visual cortex, which altogether are referred to as ocular dominance plasticity. First, evoked responses to visual stimulation of the closed eye are rapidly decreased, which is followed by an increase in responses to stimulation of the open eye in the binocular cortex (Frenkel and Bear, 2004). The temporal separation of these two events suggests that they are distinct processes that are likely mechanistically divergent.

TNF is required for the open eye potentiation phase of plasticity after MD (Kaneko et al., 2008). Using both single-unit recordings and intrinsic optical imaging techniques (where neural activity is assessed by changes in reflectance of the brain surface), Kaneko et al. (2008) show that TNF knockout animals lack this increase. Furthermore, cortical infusion of a soluble TNFR to block TNF signaling during deprivation phenocopies the result. This recapitulates the overall features of HSP: a homeostatic response to a decrease in synaptic input requiring TNF.

WHISKER DEPRIVATION

TNF has also been implicated in homeostatic plasticity in the somatosensory cortex. Trimming or plucking rodent whiskers to decrease input into the barrel cortex results in a rapid decrease in response to stimulation of deprived whiskers, followed by a slower increase in responses to neighboring spared whiskers when performed in a critical period of development (Glazewski and Fox, 1996), echoing plasticity in the visual cortex after MD. In the barrel cortex, however, the expression of plasticity has been studied in terms of cell type as well: regular spiking (RS) and intrinsic bursting (IB) pyramidal neurons behave differently (Greenhill et al., 2015). In layer 5 of the barrel cortex, unilateral whisker trimming deprivation leads to an initial depression of deprived whisker responses, followed by a slower potentiation in both RS and IB cells above original baseline levels. Critically, the potentiation is multiplicative, indicating that the plasticity is indeed HSP. This represents yet another instance where HSP occurs *in vivo*, mirroring experiments conducted in culture. However, if only one row of whiskers is trimmed, there is an initial decrease in deprived whisker responses only in RS cells, followed by a slower potentiation in both cell types that is not multiplicative, suggesting a more complex mechanism is at play when deprivation is not complete, perhaps involving multiple modalities of plasticity in addition to HSP. The authors also tested the TNF dependence using knockout animals of barrel cortex plasticity and found that the recovery from the initial potentiation in both cell types requires TNF. However, potentiation above baseline levels was only dependent on TNF in RS cells.

HEARING LOSS

TNF-dependent HSP has a clear role in two different sensory cortices, so it is interesting to speculate that HSP is a general response to sensory deprivation. Indeed, this has been examined in the auditory system using a model of conductive hearing loss (CHL) in adult mice (Teichert et al., 2017). In the primary auditory cortex, there is an initial decrease in responsiveness to auditory stimuli. After 3 days of CHL, there is a multiplicative increase in synaptic strength in the cortex, indicating it is the result of HSP. Additionally, recovery of responses to intense stimuli is impaired in TNF knockout animals, further implicating TNF in that potentiation.

Therefore, TNF-mediated HSP underlies the response to sensory deprivation in three different modalities, suggesting that it may be a general response to a decrease in sensory input. Furthermore, HSP is part of the response to changes in sensory experience in an intact animal, emphasizing that it is an important mechanism with biological relevance outside of the culture dish. It should be noted, however, that there are some differences in the expression of plasticity between the modalities. For example, ocular dominance plasticity after MD does not require TNF in an adult animal (Ranson et al., 2012). On the other hand, the hearing loss-induced HSP experiments described by Teichert et al. (2017) above were conducted in adult mice and required TNF for some components of the homeostatic response. While the existence of experience-dependent plasticity requiring TNF appears to be common to the modalities, the rules governing its expression may differ between cortical areas.

BEHAVIORAL RESPONSE TO ANTIDEPRESSANTS

TNF function in sensory cortex plasticity is consistent with a role in the response to decreased sensory input, which is an intuitive extension of the role of TNF in activity-induced HSP. TNF, however, seems to play a role in the behavioral response to antidepressants as well, which may point to a more complex function in that system.

Plasma levels of pro-inflammatory cytokines including TNF are elevated in patients with major depressive disorder (MDD; Dowlati et al., 2010), and polymorphisms in the TNF gene that modulate its expression may contribute to susceptibility to MDD (Cerri et al., 2009). At the molecular level, antidepressant treatment of rats results in increased glutamate receptor expression (Barbon et al., 2011) and synaptic localization (Ampuero et al., 2010). Therefore, the involvement of TNF in the mechanism of antidepressant action would be intriguing because it is established that TNF can modulate glutamate receptors.

This is indeed the case, as described in a report using TNF deficient mice in an animal model of depressive behavior (Duseja et al., 2015). Using two tests of depressive-type behavior, the forced swim test (FST) and tail suspension test (TST), the authors show that TNF is required for the amelioration of depressive phenotypes, a standard test for the efficacy of antidepressants. While wild type animals showed a decrease in immobility in both of these tests after administration of two different antidepressants, fluoxetine and desipramine, TNF KO animals showed no response until a much higher dose of antidepressant was used. Furthermore, the phenotype was recapitulated in GFAP-Cre, TNF-flox animals, which only lack TNF in astrocytes, suggesting that this cell type is responsible for the effect of TNF on antidepressant action. This is particularly intriguing, as the TNF released during HSP in entrohino-hippocampal slice cultures is also likely of astrocytic origin (Becker et al., 2013), raising the possibility that a similar homeostatic mechanism is at play during antidepressant administration. Furthermore, the fact that antidepressant administration does not have an immediate effect on depressive behaviors, but rather takes several weeks to reach efficacy, is consistent with a homeostatic process in its mechanism of action.

TNF EFFECTS ON STRIATAL FUNCTION

The striatum, which functions to process information in the basal ganglia, receives input from the cortex, brainstem, and thalamus and integrates those inputs to facilitate voluntary movement as well as integrate cognitive and motivational information. It is fundamentally different from the hippocampus and cortex, which are comprised of large numbers of excitatory neurons, in that it is made up of almost exclusively of inhibitory MSNs that form its only output (Gerfen and Wilson, 1996). As noted above, the TNF response of MSNs is inverted from that of pyramidal neurons (Lewitus et al., 2014). However, TNF still appears to function in an adaptive or homeostatic context in this structure.

ADAPTIVE RESPONSE TO STRIATAL DYSFUNCTION

Chronic administration of antipsychotic drugs such as haloperidol, which block D2 dopamine receptors, can result in extrapyramidal symptoms such as tardive dyskinesia (involuntary face movements) as a result of dysregulation of the striatal circuit responsible for movement. These symptoms are accompanied by both increased TNF levels as well as increased AMPA binding, raising the possibility of HSP-type mechanisms contributing to this pathology (Schmitt et al., 2003; Bishnoi et al., 2008). Blocking TNF in animals treated with haloperidol by using a dominant-negative form of the cytokine results in more frequent involuntary movements, indicating that when present, TNF functions to limit the effects of chronic haloperidol on the corticostriatal circuit (Lewitus et al., 2014). The authors of that study further show that this is through the endocytosis of GluA2-lacking AMPARs, which are trafficked in response to TNF. Altogether, this indicates that TNF is critical to a homeostatic process that serves to counter corticostriatal circuit perturbations.

BEHAVIORAL SENSITIZATION TO COCAINE

The administration of drugs of abuse to animals leads to an increase in striatal dopamine, which is accompanied by changes in glutamatergic transmission in the nucleus accumbens (NAc) of the striatum. More specifically, repeated administration of cocaine to rodents results in an initial decrease in AMPA/NMDA ratio in the NAc 24 h after the last injection, followed by a gradual increase in AMPA/NMDA ratio during a period of abstinence after that (Kourrich et al., 2007). Given that TNF can modulate AMPAR content in the striatum (Lewitus et al., 2014), it became an interesting possibility that TNF could be playing a role in circuit dynamics in a model of cocaine addiction.

A behavioral readout of responses to cocaine administration is the extent of sensitization to cocaine exposure. When given repeatedly, cocaine causes an increasingly large locomotor response, termed behavioral sensitization, and its expression

depends on AMPAR content in the NAc (Kalivas, 2009). In a study using a dominant-negative form of TNF, the authors find that when TNF is blocked, they observe both increased behavioral sensitization as well as an exaggerated potentiation of synapses onto D1-type MSNs with no initial depression, suggesting that TNF in this system serves to limit the effects of cocaine administration (Lewitus et al., 2016). Furthermore, the source of TNF in this model is microglia, as this result can be phenocopied by carrying out the same experiment in CX3CR1-Cre, TNF-flox mice which lack TNF only in microglial cells. Thus, TNF is placed within another adaptive pathway that serves to limit changes in striatal circuitry.

MORPHINE WITHDRAWAL

TNF appears to play a role in the response to other drugs of abuse in addition to cocaine. In morphine withdrawal models, TNF plays a role in synaptic adaptations after cessation of drug administration (Valentinova et al., 2019). These changes occur in the lateral habenula, an area that both processes aversive stimuli and regulates monoaminergic systems. TNF is only slightly elevated by morphine administration but increases dramatically during withdrawal. Valentinova et al. (2019) find that during withdrawal, there is a decrease in synaptic strength (as measured by AMPA/NMDA ratios) in the medial aspect of the lateral habenula, specifically in raphe-projecting neurons, which requires neuronal TNFR1 signaling. Further downstream, increased TNF signaling results in decreased sociability that is a hallmark of withdrawal symptoms. That excitatory neurons in this system have a TNF-mediated decrease in synaptic strength suggests that neurons cannot simply be divided into excitatory vs. inhibitory neurons to determine the direction of TNF-mediated changes and that the property of individual subtype of neurons (excitatory and inhibitory) must be directly tested. The work also suggests that while TNF reduces circuit changes in the NAc during drug administration, it may drive changes in other parts of the reward system, so effects across the whole circuit must be considered.

CONCLUSIONS

TNF is well known to have pleiotropic effects, allowing it to coordinate many functions under different circumstances and conditions. Within the immune system, various cell types will respond in distinct ways to coordinate the inflammatory response. We suggest that TNF may play a similar pleiotropic role in regulating neuronal circuit function. It is clear that the effects of TNF on neurotransmission are neuronal subtype-specific, and that it can lead to several different outcomes at the level of synapses. However, the common thread is that these changes still appear to normalize circuit output in response to perturbing stimuli, which is consistent with TNF being a mediator of HSP. Thus, TNF-induced trafficking of neurotransmitter receptors in the CNS may be a general mechanism by which circuit homeostasis and function are maintained both *in vitro* and *in vivo*. Disrupting TNF signaling can, therefore, be a route to investigating the role of HSP *in vivo*. However, TNF-mediated changes can also be driving changes in circuit function, as seen during morphine withdrawal. TNF-mediated HSP could also become dysregulated under pathological conditions, leading to TNF driving maladaptive changes in circuit function. Whether TNF is acting in an adaptive or maladaptive manner must be assessed for individual circuits in response to particular situations.

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RH and DS wrote the manuscript.

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Homeostatic Depression Shows Heightened Sensitivity to Synaptic Calcium

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Synapses and circuits rely on homeostatic forms of regulation in order to transmit meaningful information. The *Drosophila melanogaster* neuromuscular junction (NMJ) is a well-studied synapse that shows robust homeostatic control of function. Most prior studies of homeostatic plasticity at the NMJ have centered on presynaptic homeostatic potentiation (PHP). PHP happens when postsynaptic muscle neurotransmitter receptors are impaired, triggering retrograde signaling that causes an increase in presynaptic neurotransmitter release. As a result, normal levels of evoked excitation are maintained. The counterpart to PHP at the NMJ is presynaptic homeostatic depression (PHD). Overexpression of the *Drosophila* vesicular glutamate transporter (VGlut) causes an increase in the amplitude of spontaneous events. PHD happens when the synapse responds to the challenge by decreasing quantal content (QC) during evoked neurotransmission—again, resulting in normal levels of postsynaptic excitation. We hypothesized that there may exist a class of molecules that affects both PHP and PHD. Impairment of any such molecule could hurt a synapse's ability to respond to any significant homeostatic challenge. We conducted an electrophysiology-based screen for blocks of PHD. We did not observe a block of PHD in the genetic conditions screened, but we found loss-of-function conditions that led to a substantial deficit in evoked amplitude when combined with VGlut overexpression. The conditions causing this phenotype included a double heterozygous loss-of-function condition for genes encoding the inositol trisphosphate receptor (IP_3R —*itpr*) and ryanodine receptor (*RyR*). IP_3Rs and RyRs gate calcium release from intracellular stores. Pharmacological agents targeting IP_3R and RyR recapitulated the genetic losses of these factors, as did lowering calcium levels from other sources. Our data are consistent with the idea that the homeostatic signaling process underlying PHD is especially sensitive to levels of calcium at the presynapse.

Keywords: synapse, homeostasis, depression, *Drosophila melanogaster*, NMJ, plasticity, neurotransmission

INTRODUCTION

Animal nervous systems use forms of homeostatic synaptic plasticity to maintain stable function. Over the last 20–25 years, studies from diverse systems have revealed a wealth of information about how forms of homeostatic synaptic plasticity are implemented (Marder and Goaillard, 2006; Turrigiano, 2008; Pozo and Goda, 2010; Davis, 2013; Davis and Müller, 2015; Delvendahl and Müller, 2019). In particular, work using the *Drosophila melanogaster* neuromuscular junction (NMJ) has uncovered many facets of homeostatic implementation on a molecular level (Frank, 2014a; Frank et al., 2020). Much of the NMJ homeostasis work in both *Drosophila* and vertebrates has focused on a form of homeostatic plasticity termed presynaptic homeostatic potentiation (PHP). With PHP, manipulations that impair postsynaptic muscle receptor function trigger an increase in presynaptic vesicle release (Cull-Candy et al., 1980; Petersen et al., 1997; Davis et al., 1998; Frank et al., 2006; Wang et al., 2016).

Homeostatic plasticity at the NMJ is a bi-directional process. First, PHP is reversible—when manipulations that impair muscle receptor function are removed, the presynaptic potentiation ceases (Wang et al., 2016; Yeates et al., 2017). Second, the *Drosophila* NMJ can depress quantal content (QC) in a homeostatic manner functionally opposite to PHP: presynaptic homeostatic depression (PHD). Experimentally, one way to trigger PHD is to overexpress the *Drosophila* vesicular glutamate transporter gene, *VGlut*, in motor neurons. Overexpression of the glutamate transporter leads to an increase in the diameter of glutamatergic vesicles, an increase in quantal size across the entire distribution of spontaneous miniature events, and very large spontaneous quantal events (Daniels et al., 2004). To compensate for this, quantal content at the NMJ is lowered, resulting in normal evoked postsynaptic excitation (Daniels et al., 2004).

Many genes have been shown to be necessary for PHP at the NMJ. But much less is known about PHD. Both PHP and PHD result in opposite changes in quantal content, and studies suggest divergent and separable mechanisms governing these forms of homeostatic plasticity. Some genes required for homeostatic potentiation are dispensable for homeostatic depression (Marie et al., 2010; Gaviño et al., 2015; Li et al., 2018). Moreover, unlike homeostatic potentiation, homeostatic depression does not appear to involve a change in the size of the readily releasable pool of synaptic vesicles (Li et al., 2018). Rather, homeostatic depression appears to involve a decrease in release probability (Gaviño et al., 2015). Finally, PHP at the NMJ appears to be a process that is dependent on the input (i.e., the type of synapse formed at the NMJ; Newman et al., 2017) while PHD does not appear to be input specific (Li et al., 2018).

The degree of overlap between homeostatic depression and homeostatic potentiation is unknown. We designed a small-scale, directed screen to test for links between these two forms of homeostatic plasticity. For the screen, we targeted genes based on prior evidence that their impairment in the neuron caused a failure of the long-term maintenance of PHP. We examined loss-of-function conditions for these genes in a VGlut overexpression background for PHD. We did not find any cases of failed homeostatic depression—the conditions we examined showed decreases in quantal content in response to increased quantal size. However, we found an interesting and unexpected evoked neurotransmission phenotype: a robust decrease in excitatory postsynaptic potential (EPSP) amplitude in a VGlut-overexpressing genetic background. We observed this phenotype for a double heterozygous loss-of-function condition for the Ryanodine and IP_3 receptor-encoding genes. In our follow-up work, pharmacology phenocopied this genetic result, and our overall findings are consistent with the idea that the PHD system may show a heightened sensitivity to low calcium.

Our findings highlight a novel synaptic transmission phenotype. Prior characterizations of homeostatic depression do not report decreases in EPSP amplitude in VGlut overexpression relative to controls (Daniels et al., 2004; Marie et al., 2010; Gaviño et al., 2015; Li et al., 2018). Studies at the NMJ have resulted in models in which homeostatic compensation maintains evoked neurotransmission at the synapse approximately at control levels (Davis, 2013). Our results suggest that impairing store calcium channels may result in a cumulative defect in neurotransmission when there is a concurrent PHD challenge. We find this interesting, especially in light of the fact that these same store channels are required for the maintenance of PHP (James et al., 2019) and because other recent studies in other systems have implicated store calcium in presynaptic release mechanisms (e.g., de Juan-Sanz et al., 2017).

MATERIALS AND METHODS

Drosophila Stocks and Husbandry

Fruit fly stocks were obtained from the Bloomington Drosophila Stock Center (BDSC, Bloomington, Indiana), Kyoto Stock Center (DGRC, Kyoto, Japan), Japan National Institute of Genetics (Mishima, Shizuoka, Japan), Vienna Drosophila Research Center (VDRC, Vienna, Austria), or from the labs that generated them. w^{1118} was used as a wild-type (WT) control (Hazelrigg et al., 1984). RNAi lines and mutants used in the screen are reported in **Supplementary Table 1**.

Fruit flies were raised on cornmeal, molasses, and yeast medium (see BDSC website for standard recipe) in temperature-controlled conditions. Animals were reared at 25°C until they reached the wandering third instar larval stage, at which point they were selected for electrophysiological recording. *UAS-VGlut* (Daniels et al., 2004) was recombined with *OK371-GAL4* (Mahr and Aberle, 2006; Meyer and Aberle, 2006) to drive constitutive overexpression of VGlut. The full genotype of these animals is: *w; VGlut, OK371-Gal4/CyO-GFP*. Virgins of these flies were crossed to RNAi lines or mutants to test for changes to homeostatic depression. *w; OK371-Gal4/+* was used as a genetic control for baseline electrophysiology.

Electrophysiology and Analysis

Larvae were dissected in a modified HL3 saline comprised of: NaCl (70 mM), KCl (5 mM), $MgCl_2$ (10 mM), $NaHCO_3$

(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, except as noted).

For pharmacology, Dantrolene (R&D Systems) and Xestospongin C (Abcam) were used. Dantrolene was mixed into saline to a final concentration of 25 μM. Larvae were cut open on the dorsal side and allowed to incubate in the Dantrolene saline for 5 min. The rest of the dissection and recording was completed in Dantrolene saline. Xestospongin C was applied in a similar manner, with the animals allowed to incubate in 20 μM Xestospongin C saline for 5 min before they were recorded, also in saline containing Xestospongin C.

Electrophysiological data were collected using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA) in bridge mode, digitized using a Digidata 1440A data acquisition system (Molecular Devices), and recorded with pCLAMP 10 acquisition software (Molecular Devices). 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 excitatory postsynaptic potential (mEPSP) amplitude per NMJ was quantified by hand, approximately 100 individual spontaneous release events per NMJ (MiniAnalysis, Synaptosoft, Fort Lee, NJ, USA). Measurements from all NMJs of a given condition were then averaged. For evoked neurotransmission, 30 excitatory postsynaptic potentials (EPSPs) were averaged to find a value for each NMJ. These were then averaged to calculate a value for each condition. QC was calculated by the ratio of average EPSP and average mEPSP amplitudes for each individual NMJ. An average quantal content was then calculated for each condition. EPSP variability was assessed by measuring each of the 30 traces individually and calculating a standard deviation, coefficient of variation, and range for that NMJ. Range was defined as the maximum EPSP value minus the minimum EPSP value.

Immunostaining

An immunostaining experiment is detailed in **Figure 4**. Procedures match those previously published (Brusich et al., 2015, 2018; Spring et al., 2016; Yeates et al., 2017; James et al., 2019). Briefly, third instar larvae were filleted and fixed for 5 min with Bouin's fixative (Ricca Chemical, Arlington, TX, USA). After washes, fixed fillets were incubated in primary antibodies overnight at 4°C, mouse anti-Brp (nc82, 1:250, University of Iowa Developmental Studies Hybridoma Bank; Wagh et al., 2006) and rabbit anti-Dlg (1:5,000; Budnik et al., 1996). After washes, fillets were incubated in fluorophore-conjugated secondary antibodies overnight at 4°C (Jackson ImmunoResearch Labs, West Grove, PA, USA), goat anti-mouse-488 (DyLight, 1:1,000) and goat anti-rabbit-549 (DyLight, 1:2,000). After washes, fillets were mounted and Dlg boutons were counted blinded by hand on an epifluorescence microscope and double checked for Brp signal in apposition. Note: relative bouton numbers between NMJs 6/7 on segment A2 and A3 are consistent with earlier studies, though some raw numbers appear slightly lower, which may be due either to hand counting (rather than automated) or due to Dlg signal bouton counting (rather than HRP signal counting).

Statistical Analyses

Statistical analyses were conducted using GraphPad Prism software. Statistical significance was assessed either by Student's *t*-test when one experimental dataset was being directly compared to a control dataset, or one-way ANOVA with Tukey's *post-hoc* test when multiple datasets were being compared. For **Figure 5**, statistical tests were run as a two-way ANOVA with Tukey's *post-hoc* to test the effects of both genotype and Dantrolene application. Specific *p*-value ranges are noted in the Figure legends and shown in graphs as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ (* and $^{\#}$are used in Figures if there are additional comparisons highlighted). For some comparisons that are close to $p < 0.05$ statistical significance but do not achieve it ($0.05 < p < 0.1$), specific values are reported on the graph itself. Calcium cooperativity data were analyzed using a non-linear fit regression analysis on GraphPad Prism.

RESULTS

A Recombinant Line to Analyze Presynaptic Homeostatic Depression (PHD)

Using previously published reagents, we generated a fly stock with constitutive *VGlut* transgene overexpression. Such a stock could be used as a tool for a single-cross genetic screen. To generate the stock, we recombined the *OK371-Gal4* motor neuron driver (Mahr and Aberle, 2006; Meyer and Aberle, 2006) with a *UAS-VGlut* transgene (Daniels et al., 2004). We placed these two genetic elements in *cis* on *Drosophila melanogaster* Chromosome II. *OK371-Gal4* is an enhancer trap line for the *VGlut* promoter itself. This ensured that GAL4-driven *UAS-VGlut* overexpression would happen in desired tissues, *Drosophila* motor neurons.

We tested if the recombinant line constitutively overexpressing *UAS-VGlut* could express PHD at the NMJ. We crossed the recombinant stock to our wild-type stock (w^{1118}, herein: WT; Cross result, herein: "*VGlut, OK371/+*"). By NMJ electrophysiology, we recorded from WT control, *OK371/+* control, and *w; VGlut, OK371/+*. As expected, *VGlut, OK371/+* NMJs showed an increase in spontaneous miniature excitatory postsynaptic potential (mEPSP) amplitude compared to controls (**Figures 1A–C**; data also in **Supplementary Table 1**). Compared to WT control NMJs, there was no significant difference in evoked postsynaptic amplitudes for *VGlut, OK371/+* NMJs (**Figure 1D**; $p = 0.82$, one-way ANOVA). This was because of an accurate homeostatic decrease in QC (**Figure 1E**)—hence, successful PHD. This result matched earlier studies that had used WT as a control and a *trans OK371/UAS-VGlut* combination to induce PHD (Daniels et al., 2004; Gaviño et al., 2015; Li et al., 2018).

Even though PHD was successful relative to WT for our test cross, we noted a small, but statistically significant, baseline increase in the EPSP amplitude of *OK371/+* NMJs. This increase in *OK371/+* EPSP level was present compared either to WT control or to *VGlut, OK371/+* (**Figure 1D**). One possibility is that the *OK371/+* genetic background has slightly elevated

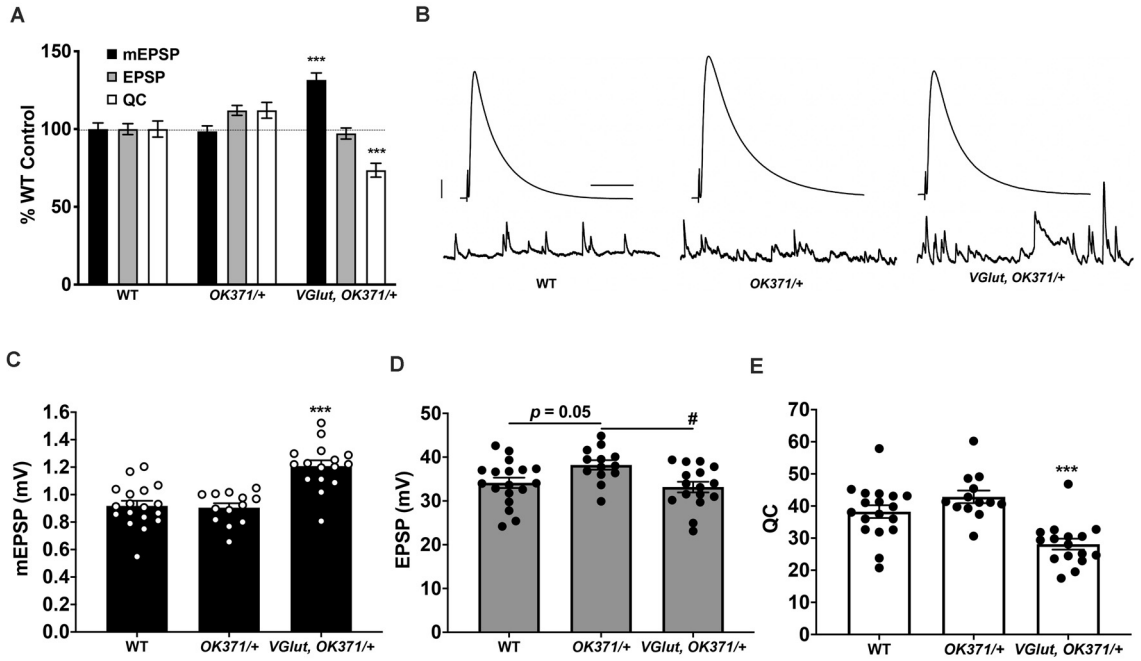

FIGURE 1 | Presynaptic homeostatic depression (PHD) works successfully with a recombinant line of *OK371-Gal4* and *UAS-VGlut*. **(A)** Neuromuscular junction (NMJ) electrophysiological data for miniature excitatory postsynaptic potentials (mEPSP), excitatory postsynaptic potentials (EPSP), and quantal content (QC). Data are normalized to wild-type (WT; w^{1118}) values. *VGlut, OK371/+* NMJs have increased mEPSP but normal EPSP because of decreased QC, indicative of successful PHD (***$p < 0.001$ vs. WT by one-way ANOVA with Tukey's *post-hoc*). **(B)** Representative electrophysiological traces. Large traces are EPSPs; small traces are mEPSPs. Scale bars for EPSPs (mEPSPs) are 5 mV (1 mV) and 50 ms (1,000 ms). **(C)** Raw data for mEPSPs. **(D)** Raw data for EPSPs. **(E)** Raw data for QC. For **(C–E)**, bars are averages and error bars are ± SEM. ***$p < 0.001$ vs. WT or vs. *OK371/+*; $^{\#}p < 0.05$ vs. *OK371/+*; analyses by one-way ANOVA with Tukey's *post-hoc*.

release, and the combined addition of *UAS-VGlut* reveals a slight depression in evoked amplitude. Noting this potentially important difference in our driver control, we continued using the *OK371/+* heterozygous condition as a genetic background control. *OK371/+* is a closer genetic control for PHD analysis than WT.

A Genetic Screen Identifies an Interaction Between Calcium Stores and a PHD-Inducing Challenge

We used our recombinant line to conduct a genetic screen for conditions that affect PHD. We crossed this stock to screen stocks: (1) either to drive *UAS-RNAi* transgenes to knock down genes; (2) to drive other chosen *UAS* transgenes; or (3) to combine with heterozygous loss-of-function mutant lines ("Materials and Methods" section, **Figure 2A**). For the screen, we targeted a subset of genes previously identified as in the neuron for homeostatic potentiation, or closely related genes. We tested 43 genotypes (sometimes multiple conditions for a single gene), including our homeostatic depression condition, *VGlut, OK371/+* (**Figures 2B,C**).

The aggregate results of the screen are reported here (**Figures 2B,C**; raw data in **Supplementary Table 1**). We recorded from 42 experimental heterozygous *mutant/+* or *>UAS-RNAi* or *UAS-transgene/+* conditions, in the *VGlut, OK371/+* genetic background. Of those 42, 12 achieved EPSPs that were numerically larger than *VGlut, OK371/+*, and 22 achieved QCs that were numerically larger than *VGlut, OK371/+* (**Figures 2B,C**). Increased evoked potentials could signify failed PHD—however, none of these cases represented statistically significant increases compared to *VGlut, OK371/+*. None were so much bigger that they were good candidates for "failed PHD." Indeed, all of the candidates had average EPSP and QC levels below *OK371/+* NMJ baseline recordings (compare **Figures 1D,E**, **2B,C**).

We recognize limitations in this kind of screening analysis. For example, we expect a certain degree of negatives or false negatives for any screen. In our case, there could be false negatives due to a limited scope of examination, the effects of non-linear summation by measuring large synaptic voltages, or due to varying baseline parameters from genotype to genotype (**Supplementary Table 1**).

Despite the negative results, we noted a phenotype distinct from what we were initially seeking: two crosses yielded larvae with striking decreases in NMJ EPSP amplitudes, more than two standard deviations below the average EPSPs from the baseline *VGlut, OK371/+* dataset (**Figure 2B**). One case was knockdown of the *Survival motor neuron* (*Smn*) gene with the *UAS-Smn[RNAi]*^{JF02057} line in the *VGlut, OK371/+* background. This was intriguing because *Drosophila Smn* is homologous to human *SMN*. Defects in *SMN* cause Spinal Muscular Atrophy (Lefebvre et al., 1995). *Drosophila Smn* has been characterized

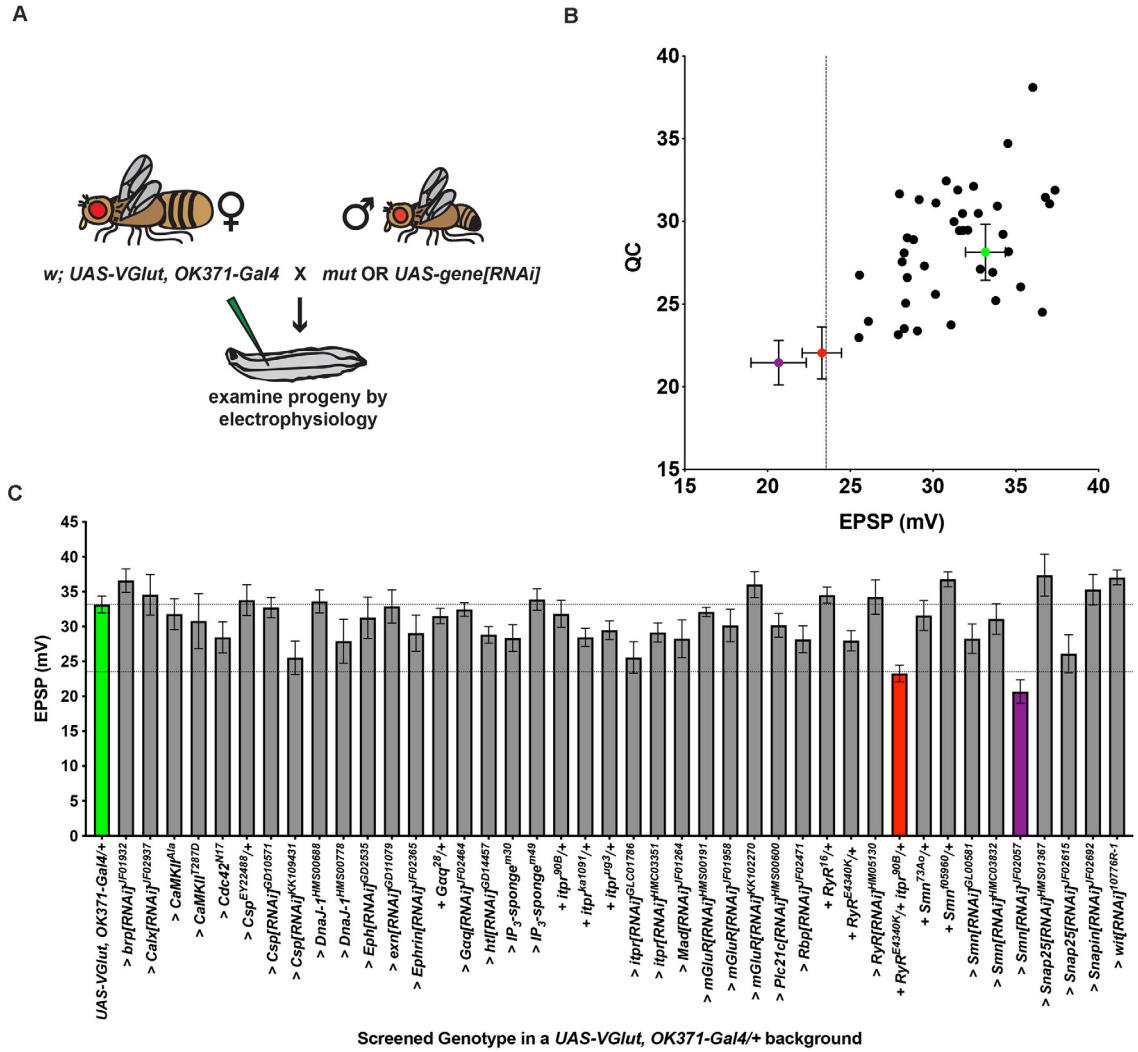

FIGURE 2 | An electrophysiology screen in a PHD-challenged genetic background. **(A)** Crossing scheme for generating larvae for electrophysiological recording. Each animal recorded had a homeostatic challenge provided by VGlut overexpression and a concurrent heterozygous or RNAi condition. Fly artwork reproduced from Brusich et al. (2015) under a Creative Commons Attribution License. **(B)** Data distribution for screened conditions (x-axis = average EPSP for condition; y-axis = average QC for condition). Green = *UAS-VGlut, OK371-Gal4/+*. Red = *UAS-VGlut, OK371-Gal4/RyR*^{E4340K}; *itpr*$^{90\,B}$/+. Purple = *UAS-VGlut, OK371-Gal4/+; UAS-Smn[RNAi]*^{JF02057}/+. Dotted line: EPSP value two standard deviations below *UAS-VGlut, OK371-Gal4/+* chosen as a cut off for potential follow-up hits. **(C)** Average EPSPs for screened conditions. All conditions have a *UAS-VGlut, OK371-Gal4/+*genetic background. ">" denotes as *UAS* construct or RNAi line being driven in motor neurons by *OK371-Gal4*. "+" denotes additional mutations present as heterozygotes. Top dotted line denotes *UAS-VGlut, OK371-Gal4/+* average. Bottom dotted line denotes two standard deviations below *UAS-VGlut, OK371-Gal4/+* average.

as a potential model for Spinal Muscular Atrophy (Sen et al., 2011; Spring et al., 2019; Raimer et al., 2020). *Smn* has also previously been implicated in PHP (Sen et al., 2011). However, the result for *UAS-Smn[RNAi]*^{JF02057} was not replicated by other *Smn* knockdown or loss-of-function mutant test crosses (**Figures 2B,C**).We did not follow up on *Smn* for this study.

A second case with a striking decrease in EPSP amplitude in the screen was a double heterozygous genetic condition in genes encoding the *Drosophila* Ryanodine receptor (*RyR*) and inositol 1,4,5-trisphosphate (IP_3) receptor (*itpr*): *VGlut, OK371/RyR*^{E4340K}; *itpr*^{90B}/+ (**Figures 2B,C**). Ryanodine receptors (RyRs) and IP_3 receptors (IP_3Rs) are localized to the endoplasmic reticulum. They mediate release of calcium from intracellular stores (Berridge, 1984, 1987, 1998; Simkus and Stricker, 2002). The *RyR*^{E4340K} mutation is a single amino acid substitution (glutamic acid to lysine; Dockendorff et al., 2000), and the *itpr*^{90B} mutation is a null mutant generated by imprecise excision of a transposon (Venkatesh and Hasan, 1997). We previously defined roles for RyR, IP_3R, IP_3 signaling and upstream components in maintaining PHP (Brusich et al., 2015; James et al., 2019).

In parallel, we screened single mutant manipulations for both genes. Neither the *RyR*^{E4340K}/+ heterozygous condition, nor the *itpr*^{90B}/+ heterozygous condition—nor any single heterozygous

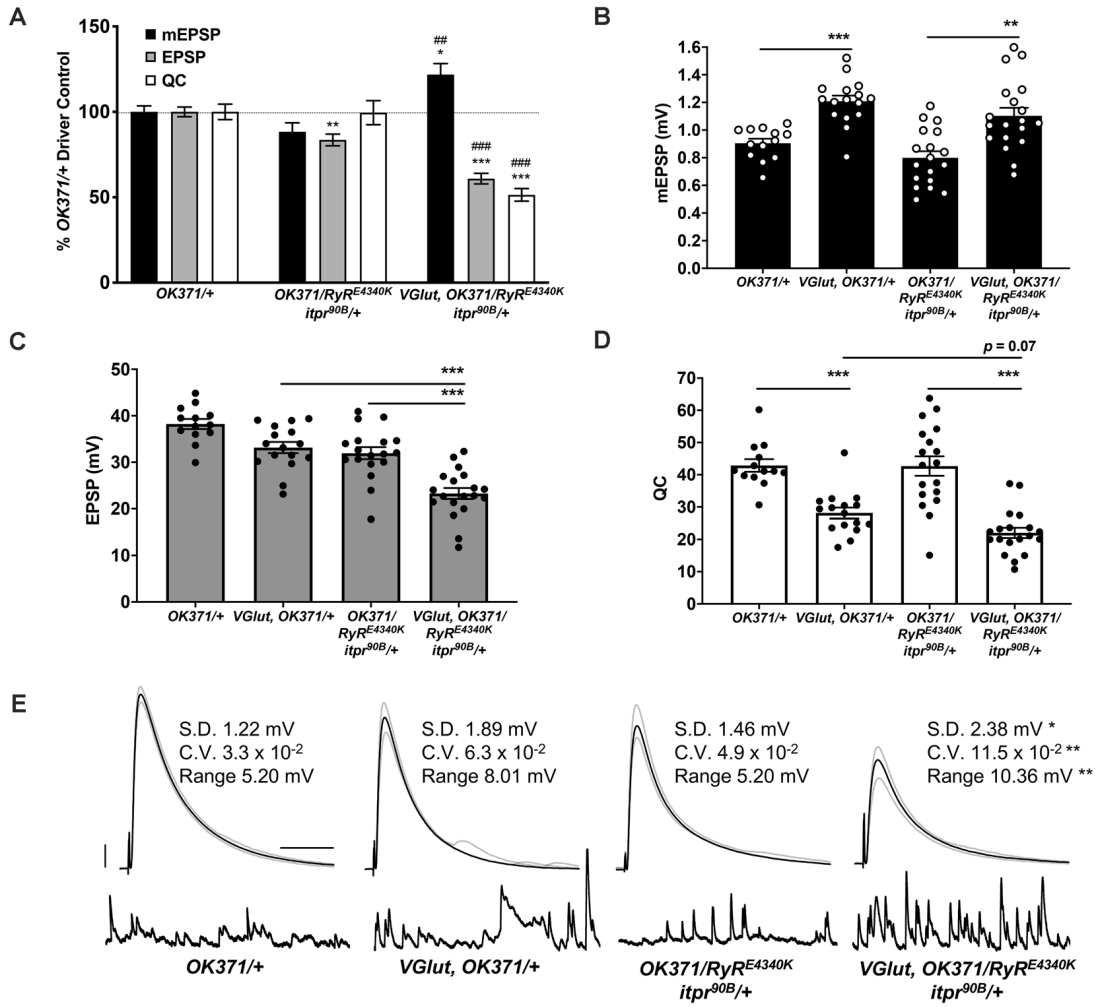

FIGURE 3 | Double heterozygous loss of the *itpr* and *RyR* genes interacts with the PHD challenge to diminish neurotransmission. Note: traces and data for *OK371/+* and *VGlut, OK371/+* are repeated from **Figure 1**, for genetic background comparison. Abbreviations are as in **Figure 1**. **(A)** NMJ electrophysiological data for mEPSP, EPSP, and QC. Data are normalized to *OK371/+* values. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ vs. *OK371/+*; $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ vs. *OK371/RyR^{E4340K}*; *itpr^{90B}/+*; analyses by one-way ANOVA with Tukey's *post-hoc*. **(B)** Raw data for mEPSPs. **(C)** Raw data for EPSPs. **(D)** Raw data for QC. For **(B–D)**, bars are averages and error bars are ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ by one-way ANOVA across genotypes, with Tukey's *post-hoc*. **(E)** Representative electrophysiological traces with standard deviation (SD), coefficient of variation (CV) and range values for EPSPs. The SD, CV, and range were significantly higher for *VGlut, OK371/RyR^{E4340K}; itpr^{90B}/+* vs. its genetic control, *OK371/RyR^{E4340K}; itpr^{90B}/+*. $^{*}p < 0.05$, $^{**}p < 0.01$ by one-way ANOVA across genotypes, with Tukey's *post-hoc*. Scale bars as in **Figure 1**.

or RNAi knockdown condition for either gene—yielded as significantly depressed EPSPs in response to PHD challenge (**Figures 2B,C**). Therefore, the screen result with the double heterozygote could be due to a genetic interaction, or it could be due to other factors in the genetic background. This preliminary finding required further characterization.

We tested if the electrophysiological phenotype could be due to a baseline neurotransmission defect when both genes are heterozygous. By electrophysiology, we compared NMJs from *OK371/RyR^{E4340K}; itpr^{90B}/+* larvae as a baseline double heterozygous condition vs. NMJs from *VGlut, OK371/RyR^{E4340K}; itpr^{90B}/+* larvae (**Figures 3A–D**). Just like WT, the baseline double heterozygous condition did have a slight decrease in EPSP amplitude compared to *OK371/+* driver control (**Figure 3A**). This indicated a small, but discernible defect in neurotransmission in animals where the IP_3Rs and RyRs are both impaired. The double heterozygous condition with concurrent *VGlut* gene overexpression showed a further decrease in transmission—compared to its own genetic control, it had increased quantal size (**Figure 3B**), but significantly decreased evoked amplitude (**Figure 3C**) because of a large decrease in quantal content (**Figure 3D**). Finally, the quantal content for *VGlut, OK371/RyR^{E4340K}; itpr^{90B}/+* NMJs was numerically smaller than for *VGlut, OK371/+* NMJs (**Figure 3D**), but this latter numerical difference was not statistically significant ($p = 0.07$, one-way ANOVA).

We noted that the EPSP amplitude in individual *VGlut, OK371/RyR^{E4340K}; itpr^{90B}/+* NMJ recordings varied markedly from stimulus to stimulus. High variability could indicate unstable neuronal excitability or release. To check if evoked release events were indeed more variable, we completed additional analyses. First, we extracted the amplitude of each individual EPSP event at every NMJ recorded. From these data, we calculated the EPSP standard deviation (SD) and coefficient of variation (CV) per individual NMJ. We also calculated a range for each NMJ by subtracting the maximum EPSP measured at each NMJ from the minimum. We averaged these SD, CV, and range measures for each genotype, considering all of the individual EPSP recordings. For all of these EPSP parameters, *w; VGlut, OK371/RyR^{E4340}; itpr^{90B}/+* animals showed statistically significant higher variability compared to controls (**Figure 3E**). By contrast, double heterozygous baseline *OK371/RyR^{E4340K}; itpr^{90B}/+* NMJs did not differ significantly from *w; OK371/+* driver control NMJs ($p > 0.85$ for each measure, Kruskal–Wallis ANOVA), suggesting that the variability stems from *VGlut* overexpression in the mutant background (**Figure 3E**). *w; VGlut, OK371/+* NMJs showed numerically higher variability than *w; OK371/+*, but this was not statistically significant (**Figure 3E**, $p > 0.25$ for each measure, Kruskal–Wallis ANOVA).

Finally, we conducted immunostaining to check if any of these electrophysiological defects might correspond with defects in synaptic growth. We assessed growth by co-staining with antibodies against the postsynaptic PSD-95 homolog, DLG (Budnik et al., 1996) and the presynaptic active zone protein, BRP (Wagh et al., 2006). We counted boutons encased by anti-DLG signal and checked that these boutons were apposed by anti-BRP signal. By this analysis, we saw no significant changes in NMJ growth: neither the PHD challenge; nor the double heterozygous loss of the *RyR/+* and *itpr/+* genes; nor combining those manipulations together yielded significant numerical differences in bouton count ($p > 0.90$ for all comparisons, one-way ANOVA; **Figures 4A,B**). One caveat to these results is that we only examined these NMJs at the level of bouton count, not at the level of the abundance of specific active zone markers (as in Böhme et al., 2019; Goel et al., 2019; Gratz et al., 2019).

Pharmacology Targeting Ryanodine and IP_3 Receptors Recapitulates Loss-of-Function Genetics

We tested if the electrophysiological phenotypes we observed could be recapitulated by combining genetics and pharmacology. We started with the drug Dantrolene. Dantrolene is a RyR antagonist (Zhao et al., 2001; Vazquez-Martinez et al., 2003). In earlier work at the *Drosophila* NMJ, we found that application of Dantrolene can abrogate the long-term maintenance of PHP (James et al., 2019).

We used a sensitized *OK371/+; itpr^{90B}/+* genetic background. With this background, we could pharmacologically impair RyRs while also genetically impairing IP_3Rs. We applied 25 μM Dantrolene to: (1) *OK371/+* NMJs; (2) *VGlut, OK371/+* NMJs; (3) *OK371/+; itpr^{90B}/+* NMJs; and (4) *VGlut, OK371/+; itpr^{90B}/+* NMJs. We also compared these conditions to a set of data for genetically identical conditions without drug treatment (**Figures 5A–C**). With two-way ANOVA statistical analyses for our electrophysiological measures, we were able to account separately for genotype effects and Dantrolene effects or interactions between the two.

In the absence of drug treatment, PHD proceeded normally (**Figures 5A–C**). We noted that the untreated *OK371/+; itpr^{90B}/+* heterozygous condition had a slightly diminished evoked amplitude compared to *OK371/+* (**Figure 5B**). Therefore, the *itpr^{90B}/+* condition could be contributing some neurotransmission loss on its own. But the addition of *VGlut* transgenic expression to this heterozygous background did not further decrease evoked neurotransmission (**Figure 5B**), indicating normal PHD, as signified by an expected decrease in quantal content (**Figure 5C**).

With 25 μM Dantrolene treatment, the data were more complex. First, mEPSP amplitudes were generally smaller than

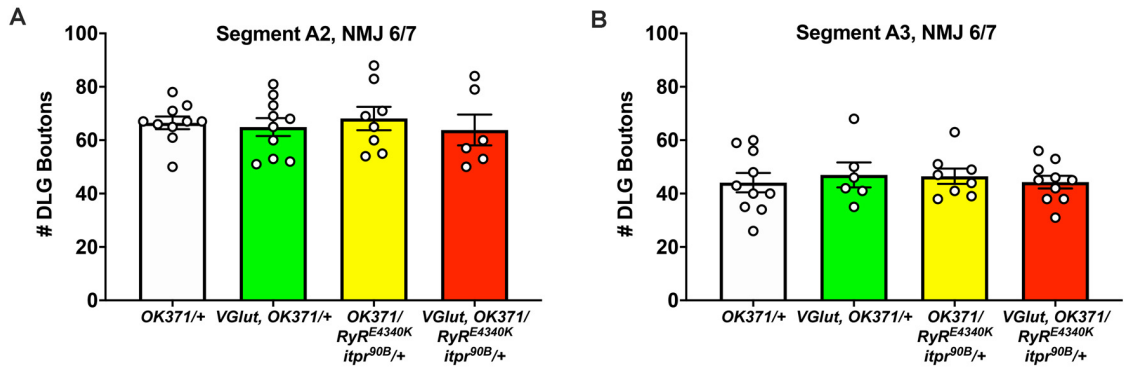

FIGURE 4 | No discernible NMJ growth defects. NMJs of third instar larvae (same genotypes as **Figure 3**) were analyzed by immunostaining, co-staining with anti-DLG for the postsynaptic density and anti-Brp to check for apposed presynaptic active zones. **(A)** DLG boutons counted for Segment A2, NMJ 6/7. **(B)** DLG boutons counted for Segment A3, NMJ 6/7. No significant differences were found across genotypes ($p > 0.9$ for every possible head-to-head comparison, one-way ANOVA).

without treatment (**Figure 5A**). Paradoxically, such a decrease could potentially trigger a short-term induction of PHP in the baseline *OK371/+* condition (**Figure 5C**)—even though a lower dose of Dantrolene actually abrogates the long-term maintenance of PHP (James et al., 2019). Yet even if this is the case, we are able to do our analysis. A prior study demonstrated that the PHP and PHD processes can occur additively at the same NMJ without interference (Li et al., 2018).

For our experiments with Dantrolene in the *VGlut*-overexpressing backgrounds, mEPSPs were elevated compared to their respective genetic controls with Dantrolene (**Figure 5A**). This indicated that in the presence of Dantrolene, *VGlut* overexpression still caused homeostatic pressure that could induce PHD. Additionally, with Dantrolene, EPSP amplitudes in *VGlut*-overexpressing lines were decreased compared to their respective genetic controls (**Figures 5B**). This was due to large decreases in quantal content (**Figure 5C**).

Interestingly, the *VGlut, OK371/+; itpr^{90B}/+* condition (+Dantrolene) had depressed evoked amplitudes compared either to the *VGlut, OK371/+* (+Dantrolene) condition ($p < 0.01$, two-way ANOVA) or to the *OK371/+; itpr^{90B}/+* (+Dantrolene) condition ($p = 0.07$, two-way ANOVA; **Figure 5B**). We note that the latter case does not achieve statistical significance on its own. However, two-way ANOVA analyses on the datasets show that both EPSP amplitudes and quantal content have a significant degree of their variation explained by an interaction between genotype and Dantrolene application (**Figure 5D**).

Collectively, our data could indicate a cumulative neurotransmission defect when impairing both the IP_3Rs and RyRs in a PHD-challenged background (electrophysiological traces, **Figure 5E**). We needed to test this idea further with more combinations and genetic conditions.

It is possible that strong impairment of RyRs could be sufficient to cause synthetic phenotypes in conjunction with the PHD regulation system. We reasoned that Dantrolene might be able to exert strong effects in a heterozygous *RyR/+* background because this is not a null *RyR* genetic condition. Therefore, we ran additional pharmaco-genetic tests using a second sensitized genetic background, *OK371/RyR^{E4340K}*—both with and without drugs and with and without *UAS-VGlut* overexpression. Again, in the absence of pharmacological treatment, PHD proceeded normally in the heterozygous

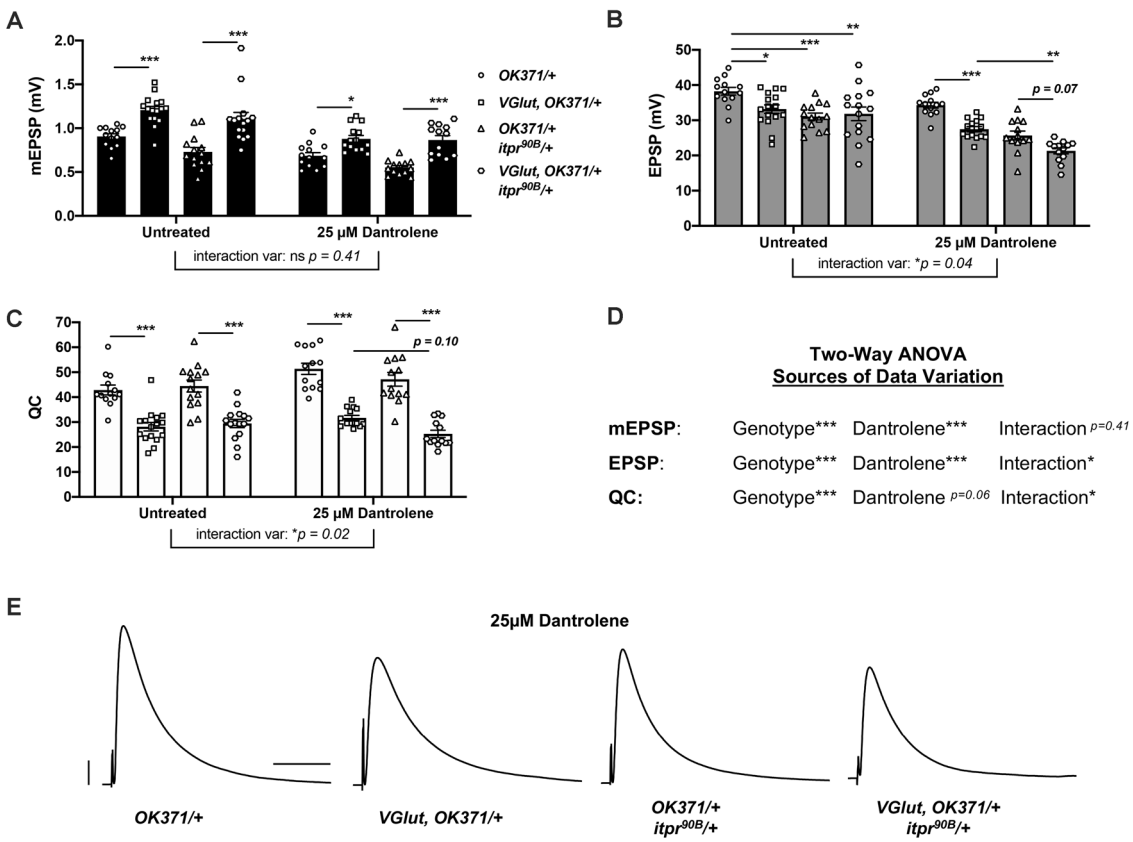

FIGURE 5 | Genetic impairment of *itpr* combined with pharmacological impairment of RyR phenocopies genetic findings. Notes: Untreated data for *OK371/+* and *VGlut, OK371/+* are repeated from **Figure 1**, for genetic background comparison, and analyses are by two-way ANOVA with Tukey's *post-hoc* to account for genotype effects, 25 µM Dantrolene effects, and interaction effects between genotype and Dantrolene. **(A)** Raw data for mEPSPs. **(B)** Raw data for EPSPs. **(C)** Raw data for QC. For **(A–C)**, bars are averages and error bars are ± SEM, with individual datapoint shapes corresponding to genotype. **(D)** Two-way ANOVA analysis of the effects of each parameter on data variation for each electrophysiological measure. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ by two-way ANOVA with Tukey's *post-hoc*. **(E)** Representative EPSP traces. Scale bars are as in **Figure 1**.

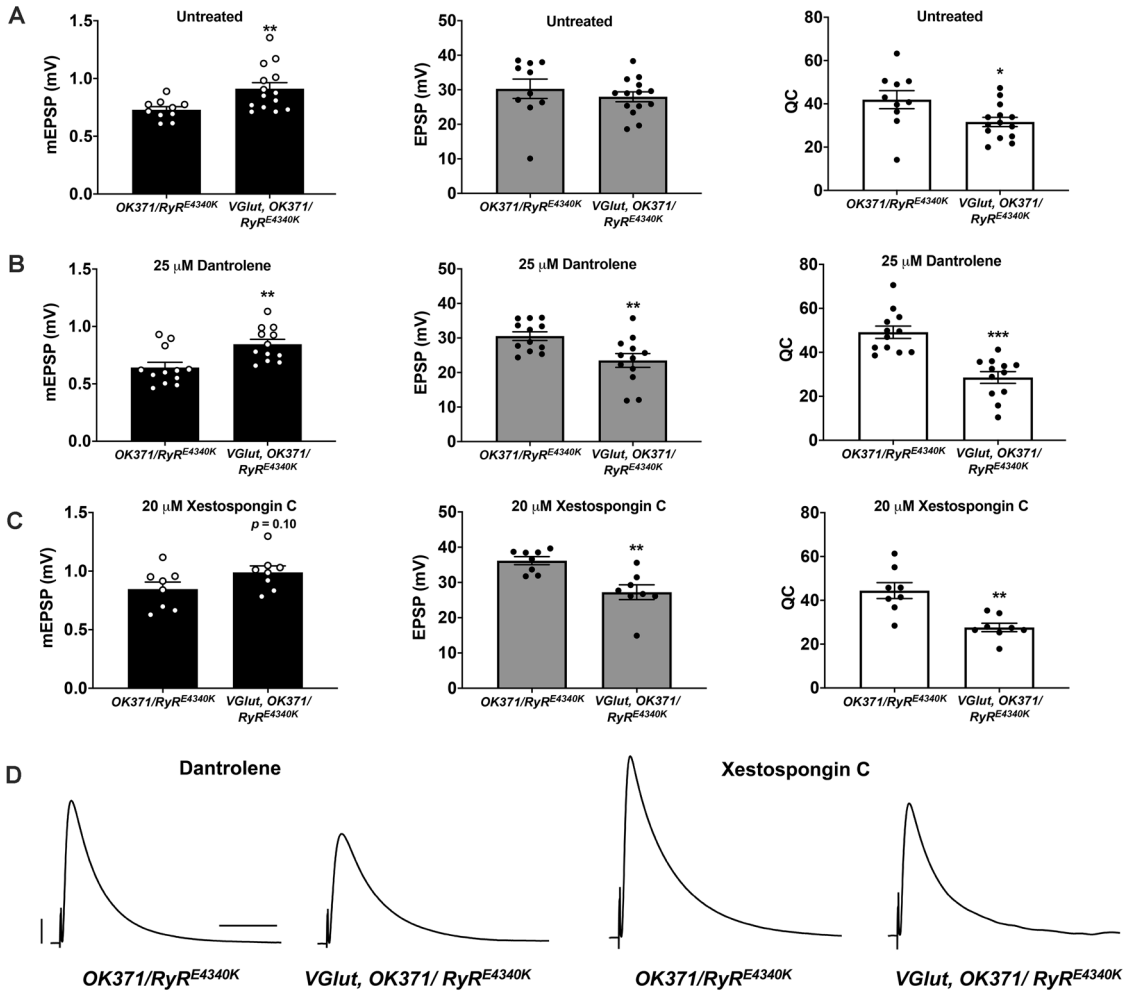

FIGURE 6 | Additional pharmaco-genetic combinations phenocopy the genetic conditions. **(A)** Raw data for mEPSPs (left); raw data for EPSPs (middle); raw data for QC (right) for untreated genotypes as shown; bars are averages and error bars are ± SEM. **(B)** Data as in **(A)** but with 25 μM Dantrolene added to NMJ preps. **(C)** Data as in **(A)** but with 20 μM Xestospongin C added to NMJ preps. **(D)** Representative EPSP traces. Scale bars are as in **Figure 1**. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ by Student's *t*-test comparing a control dataset (no VGlut overexpression) vs. an experimental dataset (VGlut overexpression).

OK371/RyR^{E4340K} genetic background (**Figure 6A**). With Dantrolene, mEPSPs became significantly larger when *VGlut* was expressed (**Figure 6B**, left), but EPSPs were significantly reduced (**Figures 6B**, middle, **6D**) because of a decrease in quantal content (**Figure 6B**, right).

Finally, we attempted the inverse pharmaco-genetic experiment from that in **Figure 5**. This time we used the IP_3R inhibitor, Xestospongin C (Gafni et al., 1997; Wilcox et al., 1998) and the sensitized *OK371/RyR*^{E4340K} genetic background. We applied 20 μM Xestospongin C, both to *OK371/RyR*^{E4340K} NMJs and to *VGlut, OK371/RyR*^{E4340K} NMJs. mEPSPs were numerically larger when *VGlut* was overexpressed (**Figure 6C**, left)—though interestingly, for the Xestospongin C dataset, the data did not achieve statistical significance for mEPSP size ($p = 0.10$, one-way ANOVA). This could indicate only weak to no homeostatic pressure in the presence of Xestospongin C. Nevertheless, EPSPs were significantly reduced (**Figures 6C**, middle, **6D**) because of a marked decrease in quantal content (**Figure 6C**, right).

Taking all of these data together, for each case where we examined a dual impairment of RyR and IP_3R the EPSP amplitudes were all quite low with concomitant VGlut overexpression (**Figures 3**–**6**).

PHD in Very Low Extracellular Calcium

We wondered how impairment of channels that mediate release of calcium from intracellular stores might cause the electrophysiological phenotypes that we observed. It could be the case that they are part of the PHD system. Or it could be the case that impairing these channels does not impinge upon PHD signaling itself—but their loss may sensitize the synapse to additional challenges, such as those brought on by PHD.

Our prior work suggested that these ER calcium store channels and the signaling systems that control them are required to maintain homeostatic potentiation throughout life (Brusich et al., 2015; James et al., 2019). We also found a related result: impairing Ca^{2+} store release mollified hyperexcitability phenotypes caused by gain-of-function Ca_V2 amino-acid substitutions in the alpha1 subunit Cacophony. Ca_V2 channels mediate synaptic calcium influx at the NMJ (Brusich et al., 2018). In light of these prior data, we considered two possibilities for PHD. One model is that the IP_3R and RyR channels play a role in ensuring proper level of neurotransmission coincident with PHD. A different model is that calcium itself plays the important role. If this latter idea were true, it might be the case that lowering calcium influx into the presynaptic terminal would also be sufficient to interact with the PHD signaling process, ultimately lowering evoked transmission.

As a test, we measured release over a range of low extracellular calcium concentrations (0.2–0.5 mM). We examined six genotypes: (1) WT; (2) *w*; *OK371/+*; (3) *w*; *VGlut, OK371/+*; (4) *w*; RyR^{E4340K}/+; $itpr^{90B}$/+; (5) *w*; *OK371/*RyR^{E4340K}; $itpr^{90B}$/+; and (6) *w*; *VGlut, OK371/*RyR^{E4340K}; $itpr^{90B}$/+. To organize data and to calculate calcium co-operativity, we plotted quantal content as a function of calcium concentration, with the x-y axes on a log-log scale (**Figures 7A,B**). To account for different Ca^{2+} driving forces in the different concentrations, we corrected QC for nonlinear summation in our plots and in our subsequent analyses (NLS Corrected QC; Martin, 1955).

Non-linear regression analyses revealed that there was no significant difference in calcium co-operativity between any of these genotypes over the range of extracellular [Ca^{2+}] we tested (**Figures 7A,B**). The calculated log-log slope values of the control PHD genotypes were: WT (log-log slope = 1.810), *w; OK371/+* (log-log slope = 1.884), and *w; VGlut, OK371/+* (log-log slope = 2.117). Comparing those three slopes with one another by nonlinear regression yielded no significant difference in slope ($p = 0.91$). The log-log slope values of the double heterozygous conditions were: *w;* RyR^{E4340K}/+; $itpr^{90B}$/+ (log-log slope = 1.737), *w; OK371/*RyR^{E4340K}; $itpr^{90B}$/+ (log-log slope = 2.102), and *w; VGlut, OK371/*RyR^{E4340K}; $itpr^{90B}$/+ (log-log slope = 1.601). Comparing those slopes with one another also yielded no significant difference ($p = 0.77$).

Even though there was no significant difference in calcium co-operativity of release over the range of low [Ca^{2+}] conditions examined, our data did show a very large drop in release between 0.3 and 0.2 mM [Ca^{2+}]—specifically for the genotypes where PHD was induced by *UAS-VGlut* overexpression, or for the genotypes with a double heterozygous impairment of *RyR* and *itpr*. Examining the raw data at 0.2 mM [Ca^{2+}], we observed that there was significant homeostatic pressure for PHD signified by mEPSP amplitude increases in the

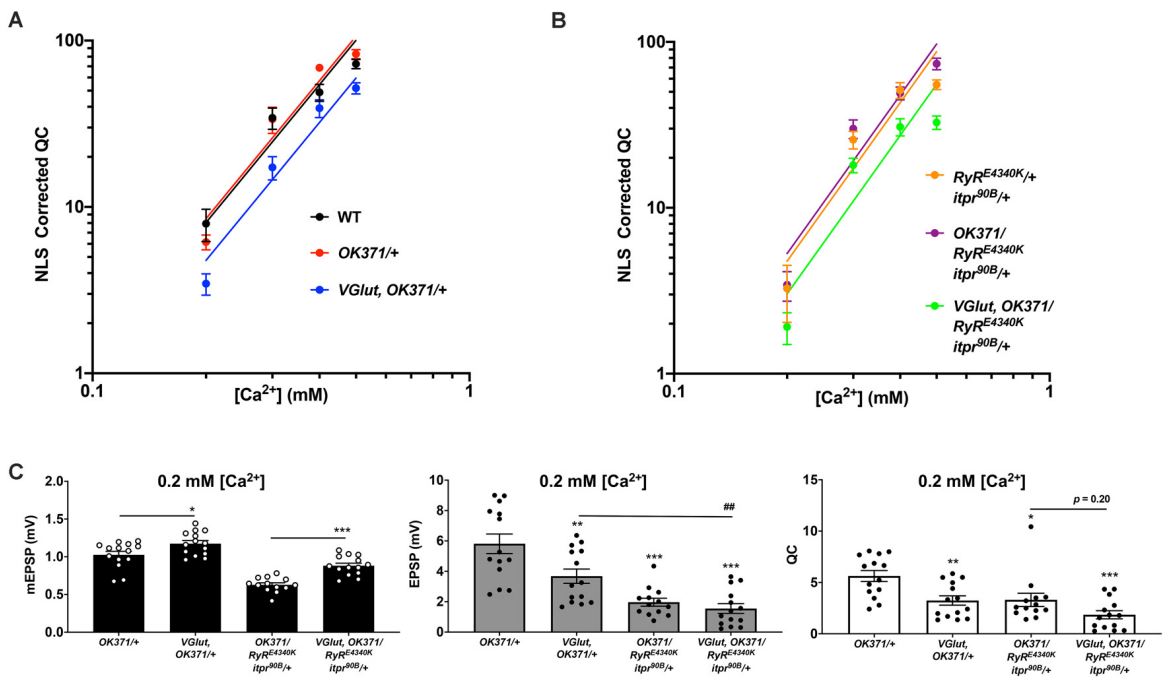

FIGURE 7 | Ca^{2+} concentration-sensitivity of PHD execution. **(A)** log-log plots of recording saline [Ca^{2+}] vs. QC corrected for non-linear summation for WT, *OK371/+*, and *VGlut, OK371/+* conditions. Across the range of [Ca^{2+}] examined, there is no significant difference in calcium cooperativity for these conditions (Nonlinear Regression, $p = 0.91$). **(B)** Data plotted as in **(A)** but this time with a double heterozygous RyR^{E4340K}/+; $itpr^{90B}$/+ genetic background. Across the range of [Ca^{2+}] examined, there is no significant difference in calcium cooperativity for these conditions (Nonlinear Regression, $p = 0.78$). **(C)** Raw data for mEPSPs (left); raw data for EPSPs (middle); raw data for QC (right). All data are for the indicated NMJ genotypes in 0.2 mM [Ca^{2+}]; bars are averages and error bars are ± SEM. For mEPSPs, $^*p < 0.05$ and $^{***}p < 0.001$ by Student's *t*-test, comparing PHD-challenged genotypes vs. unchallenged genetic controls. For EPSPs and QC, $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ vs. *OK371/+*; $^{\#\#}p < 0.01$; EPSP and QC analyses done across multiple genotypes by one-way ANOVA with Tukey's *post-hoc*.

VGlut-overexpression background (**Figure 7C**, left). Yet except for the control NMJs, EPSP amplitudes were very much diminished (**Figure 7C**, middle) because of stark drops in QC (**Figure 7C**, right).

Together, the data point to two conclusions. First, low extracellular calcium on its own appears to be a case where the synapse experiences a synergistic interaction with PHD challenge (**Figure 7C**, *VGlut, OK371/+* data). Second, double heterozygous impairment of *RyR* and *itpr* appears to cause very low levels of baseline release in low calcium, irrespective of PHD challenge (**Figure 7C**, middle; compare with **Figure 3C**). Taken together, these data suggest that lowering presynaptic calcium by any means (impairing store release and/or impairing influx) is sufficient to impair evoked levels of excitation, in conjunction with a PHD challenge.

PHD Challenge Interacts With Impaired Ca_V2 Function

As a final test, we turned back to genetics. *Drosophila* Ca_V2 channels mediate synaptic calcium influx at the NMJ. We used a hypomorphic mutant in the Ca_V2 alpha1 subunit-encoding *cacophony* gene, cac^S, to limit calcium influx. Ca_V2 is essential for viability, but cac^S hypomorphs are viable and fertile (Smith et al., 1998; Kawasaki et al., 2000). Earlier work showed that the cac^S homozygous condition dampens NMJ EPSP amplitude by about 70–80% (Frank et al., 2006); calcium imaging data suggest this is due to a ∼50% decrease in Ca^{2+} influx during evoked stimulation (Müller and Davis, 2012). Beyond this phenotype in baseline neurotransmission, cac^S hypomorphs also block PHP expression and PHP-associated increases in presynaptic calcium influx (Frank et al., 2006; Müller and Davis, 2012).

With a single cross, we generated hemizygous cac^S/Y*; VGlut, OK371/+* male larvae (**Figure 8A**). Compared to cac^S/Y as a baseline mutant control, cac^S/Y*; VGlut, OK371/+* NMJs have a marked increase in mEPSP size (**Figure 8B**), indicating homeostatic pressure to induce PHD (**Figure 8B**). However, comparing evoked potentials of those two conditions shows that cac^S/Y*; VGlut, OK371/+* NMJs have much smaller EPSPs (**Figure 8C**) and a very large decrease in QC (**Figure 8D**).

DISCUSSION

We began this study in search of genetic conditions that affect PHD (**Figure 2**). While we did not find any conditions that result in a block of PHD, we did find conditions that provide insight into how calcium regulation may interact with this form of homeostatic plasticity to affect synapse function. When IP_3R and RyR functions are partially impaired—either by genetics or by pharmacology—the NMJ still executes a PHD-like

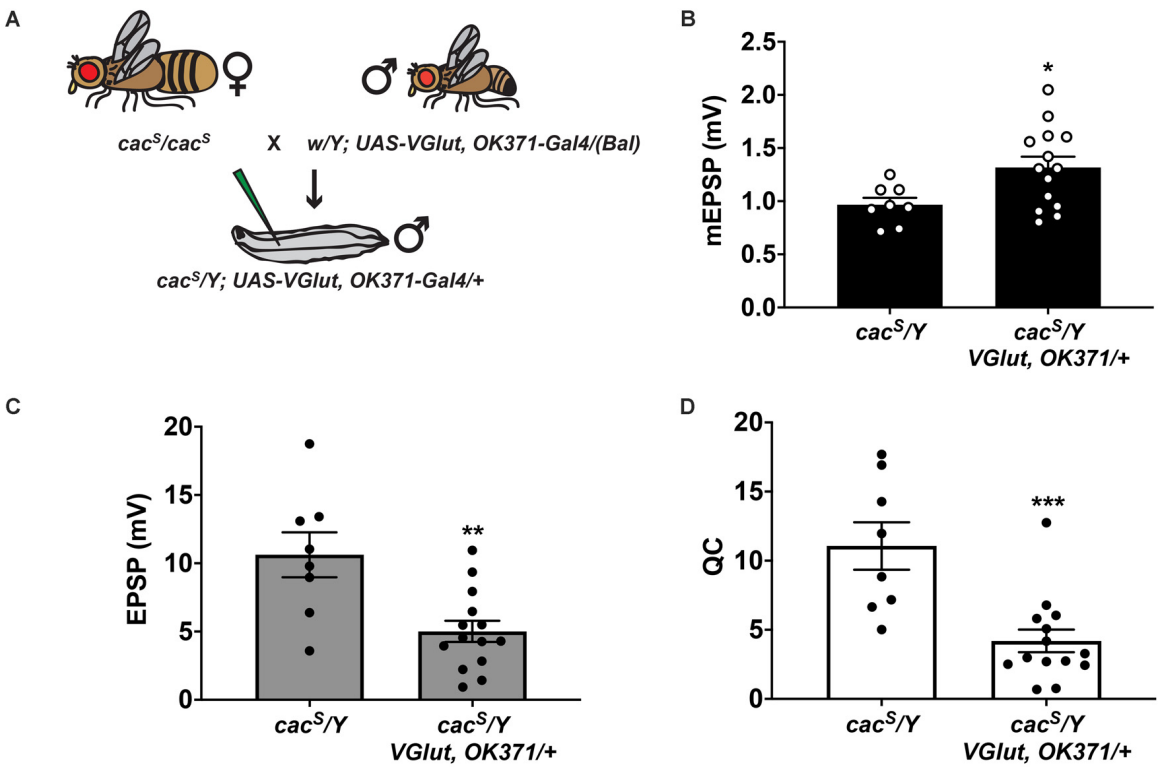

FIGURE 8 | Partial impairment of Ca_V2/Cacophony and PHD. **(A)** Crossing scheme for generating larvae for electrophysiological recording. Male larvae were hemizygous for the cac^S hypomorphic mutation. Fly artwork reproduced from Brusich et al. (2015) under a Creative Commons Attribution License. **(B)** Raw data for mEPSPs. **(C)** Raw data for EPSPs. **(D)** Raw data for QC. For **(B–D)**, bars are averages and error bars are ± SEM. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ by Student's *t*-test comparing the control cac^S dataset (no *VGlut* overexpression) vs. the experimental cac^S dataset (*VGlut* overexpression).

process. But that process goes beyond what is appropriate for the homeostatic pressure that is applied to the system. As a result, evoked potentials at the NMJ are much smaller than baseline (**Figures 3–6**). A similar phenotype is observed when extracellular [Ca^{2+}] is lowered to 0.2 mM (**Figure 7**) and when the Ca_V2 alpha1 subunit gene *cacophony* harbors a hypomorphic mutation, cac^S (**Figure 8**).

This phenotype has important implications for proper control of synapse function. Taking our data together, we propose that perturbations that dampen calcium efflux from stores or perturbations that dampen calcium influx from the extracellular environment can both synergistically interact with a PHD challenge to control levels of evoked neurotransmission (**Figure 9**).

Screen Limitations

We did not identify conditions that blocked PHD, and here we discuss potential limitations of the screen. First, our primary assay was electrophysiology, and we employed a candidate-based method, similar to what has previously been documented in the field for PHP (Frank et al., 2020). By definition, candidate-based screens are limited in scope. Second, we focused on factors previously implicated in the maintenance of PHP function (or closely related signaling factors). The idea was that some factors needed to maintain synaptic homeostasis may be needed to orient the NMJ toward a proper, physiological level of function, regardless of the nature of the homeostatic challenge. This idea could have valence, but it was not guaranteed to produce mutant conditions with greater than normal evoked amplitudes in our screen.

Regarding the electrophysiological data, we did find instances in which the screened EPSP was numerically larger than the baseline for *VGlut, OK371*/+, but no instances identified as "PHD-blocking" (**Figure 2**). The *VGlut, OK371*/+ baseline evoked potential was high (∼35 mV), so it is possible that potential positives at a higher potential could be obscured by the limits of non-linear summation. There were also variations from line to line in resting membrane potential, input resistance, and the degree of mEPSP increase indicative of PHD challenge (**Supplementary Table 1**). All of these parameters could contribute to false negatives for the screen. Unless a screen is done to saturation, there will be false negatives. It is important to interpret those parsimoniously. For our screen, we believe the way to interpret a negative is not to state that the screen definitively ruled out a factor—rather, the screen failed to rule in that factor for follow-up study.

Similarities and Differences With Prior PHD Studies at the NMJ

We were able to conduct a PHD screen using our recombinant stock with the *UAS-VGlut* and *OK371-Gal4* elements on the same chromosome. In principle, such a stock can pick up modifier mutations. The trade-off was a simplified, single-generation crossing scheme for genetic screens. Our recombinant stock with the driver and *UAS* elements in *cis* maintains consistent PHD challenge from generation to generation, and it behaves similarly electrophysiologically to *trans OK371/VGlut* combinations used in other studies (Daniels et al., 2004; Gaviño et al., 2015; Li et al., 2018).

There are differences between our study and the findings of other published work. Prior studies have used WT (or w^{1118}) as a control background when compared to VGlut overexpression (Daniels et al., 2004; Gaviño et al., 2015; Li et al., 2018). This is a standard practice. Those studies reported precise PHD when comparing WT vs. *OK371/VGlut* third instar larvae—decreased QC at *OK371/VGlut* NMJs resulting in unchanged evoked transmission. We replicated this finding (**Figure 1**). However, we also used our Gal4 driver stock background *OK371*/+ as an additional control. For that comparison, we saw a slight depression in the evoked amplitude of *OK371, VGlut*/+ NMJs (**Figure 1**). One possibility is that our recombinant stock was acting as a sensitized background.

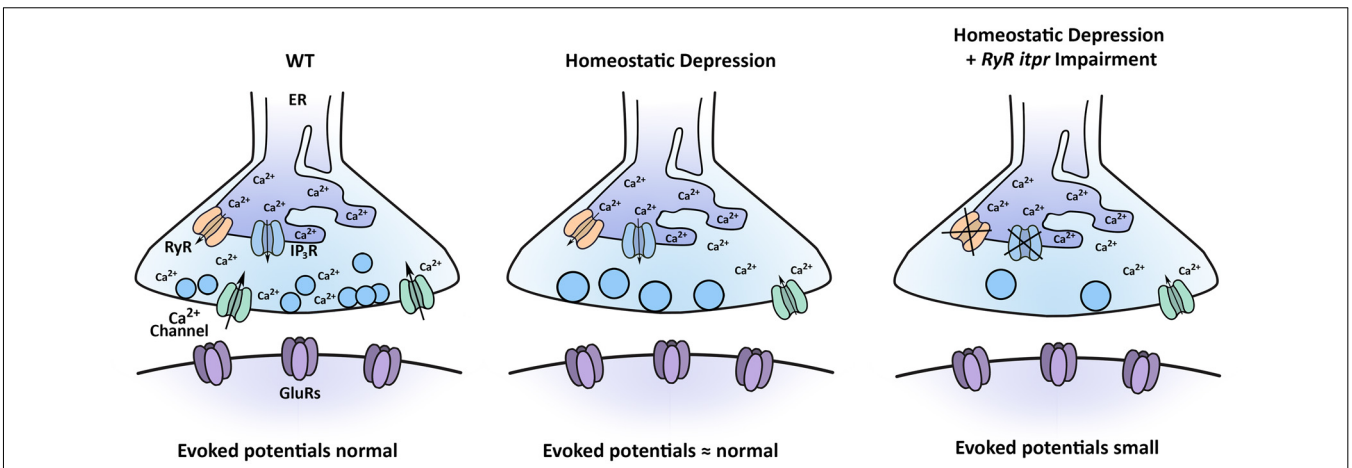

FIGURE 9 | Model for how multiple calcium sources interact with the process of PHD. Under baseline conditions, Ca_V2-type calcium channels contribute to synapse function, as may RyRs and IP_3Rs. Under conditions inducing PHD, synaptic vesicles are enlarged, and QC is decreased, through regulation of sources of calcium. When PHD challenge is coupled with concomitant impairment of RyR and IP_3R channels, evoked potentials are significantly diminished.

A second difference comes from the low extracellular calcium test. A low extracellular calcium experiment was previously done when VGlut overexpression was first characterized (Daniels et al., 2004). For that study, the authors showed that QC was significantly diminished compared to wild-type NMJs by the method of failure analysis. Taking the data of that study in aggregate, the authors concluded that PHD was intact in a variety of conditions, including saline with very low extracellular [Ca^{2+}] (0.23 mM Ca^{2+}, 20 mM Mg^{2+}). Our study may appear to conflict with that study because we found that saline with very low [Ca^{2+}] (0.2 mM Ca^{2+}, 10 mM Mg^{2+}) is conducive to an interaction with PHD, resulting in low evoked release. One possibility is that since the original study was examining failure percentage vs. WT—and not the absolute value of mEPSPs or EPSPs in low calcium, this might not be as easily observed. Other differences might be attributed to genetic background or other differences in recording saline, like magnesium concentration.

Finally, one other study previously examined the effects of a cac^S mutation with concomitant VGlut overexpression (Gaviño et al., 2015). The authors did not find the low evoked potentials that we report. The major difference between that experiment and ours is that the prior work examined the cac^S mutation in an extracellular [Ca^{2+}] (1.0 mM) that was double that of our study. The result was a Ca^{2+} driving force that yielded robust baseline EPSPs, even in the cac^S mutant background (Gaviño et al., 2015). Given our results with low calcium concentration (**Figure 7**), a similar effect may be at work here.

Known Roles for Calcium in Controlling Homeostatic Plasticity

The notion that calcium contributes to successful homeostatic signaling is not new. Many roles for voltage-gated calcium channels in synaptic homeostasis are well-documented (Frank, 2014a,b). Prior to our study, there was evidence for voltage-gated calcium channel regulation for both NMJ PHP and PHD. For PHP, loss-of-function conditions in Ca_V2/*cacophony* can impair or block this form of homeostatic regulation (Frank et al., 2006, 2009; Müller and Davis, 2012; Spring et al., 2016). Calcium imaging experiments suggest that the reason is because an increase in calcium influx through Ca_V2 is required for the upregulation of quantal content during PHP, and mutant conditions like cac^S block this increase (Müller and Davis, 2012). Recent studies report that Cacophony and other active zone protein levels increase at the NMJ active zone in response to PHP homeostatic challenges (Böhme et al., 2019; Goel et al., 2019; Gratz et al., 2019). And work from mammalian systems mirrors these findings. For example, with mouse hippocampal cultures, TTX exposure induces a homeostatic decrease in presynaptic calcium influx (Zhao et al., 2011).

The converse appears true for PHD. Calcium imaging data from two different studies has shown a decrease in the size of calcium transients at the NMJ in response to presynaptic nerve firing in VGlut-overexpressing animals (Gaviño et al., 2015; Li et al., 2018). The data are mixed on how these decreased transients might come about during PHD. Using a tagged *UAS-cacophony* cDNA transgene, two studies verified that there was a reduction in the amount of GFP-tagged Cacophony alpha1 subunits in Ca_V2 in a VGlut-overexpressing background (Gaviño et al., 2015; Gratz et al., 2019). However, one of these same studies demonstrated that if a tagged genomic construct is used instead, that same Ca_V2 reduction is not observed (Gratz et al., 2019). Since the transgenic tagged Cacophony-GFP is the product of a single *cac* splice isoform (Kawasaki et al., 2002, 2004), it could be the case that some isoforms are more dynamically trafficked at the synapse. Another possibility is that existing active zone components are somehow modulated during PHD. Regardless of the actual mechanism, the phenomenon appears conserved: again, with rodent hippocampal preparations, increased neuronal activity through gabazine exposure induces a PHD-like phenomenon ultimately resulting in decreases in calcium influx and release (Zhao et al., 2011; Jeans et al., 2017).

How Do Calcium Stores Interact With PHD?

Calcium stores have been studied in the context of neurotransmission and plasticity. We know that endoplasmic reticulum (ER) can be visualized at *Drosophila* NMJ terminals (Summerville et al., 2016), and recently developed imaging tools employed in multiple systems (including at the *Drosophila* NMJ) show how nerve stimulation results in dynamic changes to ER lumenal calcium (de Juan-Sanz et al., 2017; Handler et al., 2019; Oliva et al., 2020). In parallel, other groups working at the *Drosophila* NMJ have demonstrated important roles in baseline neurotransmission and in PHP for ER resident proteins (Genç et al., 2017; Kikuma et al., 2017). And from our prior work, we know that store calcium channels and upstream signaling components are important for maintaining the NMJ's capacity for PHP throughout life (Brusich et al., 2015; James et al., 2019). We also know that disrupting these same factors can ameliorate hyperexcitability associated with gains of Ca_V2 function (Brusich et al., 2018). Finally, from mammalian work it is clear that IP_3Rs, RyRs, and intracellular calcium govern a variety of forms of neuroplasticity (Berridge, 2016), including paired pulse facilitation (Emptage et al., 2001), and modulation of voltage-gated calcium channel activity (Lee et al., 2000; Catterall, 2011).

If PHD were simply a matter of properly functioning neurotransmission machinery, then it is not entirely obvious why PHD would be so sensitive to the amount of calcium available such that evoked release would be impaired greatly either when store-operated channels were impaired or when the amount of influx was lowered. In our study, neurotransmission has not been lowered beyond a point of synapse failure. This means that there is still functional machinery. And PHD, *per se*, is not disrupted—indeed, there is still depression.

With any type of homeostatic system, there not only needs to be error detection (large quantal size) and correction (decreased quantal content), but there also need to be brakes applied to the system to prevent some kind of overcorrection. At first glance, our data could suggest some manner of PHD "overcorrection." In our view, this is an interesting and understudied type of phenomenon that could be examined in many homeostatic systems. But it is also true that the nature of the PHD challenge

could simply represent a genetic background that renders the synapse sensitive to any additional insults.

So how exactly do levels of calcium (or the function of distinct types of calcium channels found at the synapse) ultimately affect excitation levels? This is a difficult problem. The first step might be to narrow the relevant tissue type(s) involved in PHD signaling. ER and store-operated channels are relevant to the functions of many tissues. In principle, our genetic loss-of-function manipulations to *itpr* and *RyR* could affect store-operated channels either in the neuron or in the muscle or in surrounding tissues like glia. Our pharmacological manipulations using Dantrolene and Xestospongin C could also affect multiple tissue types. Therefore, in principle, changing the levels of cytosolic calcium could either affect local signaling in the neuron, or it could result in aberrant signaling back to the presynaptic neuron, disorienting the homeostat.

We favor the idea that the relevant calcium signal is local in the motor neuron for two reasons. First, from our own data, we were able to observe the small evoked neurotransmission phenotype either with manipulations to store calcium or with manipulations that affect presynaptic calcium influx, including partial loss-of-function of neuronal *cacophony*. Second, a recent study puts forth data suggesting that when VGlut overexpression induces PHD, this happens exclusively because of excess presynaptic glutamate release, and presynaptic depression is initiated independent of any sort of postsynaptic response (Li et al., 2018). Such an autocrine signaling mechanism could very well reveal a role for intracellular calcium signaling in the presynapse.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

CJY and CAF designed research, performed research, analyzed the data, wrote, and edited the article. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fncel.2021.618393/full#supplementary-material.

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