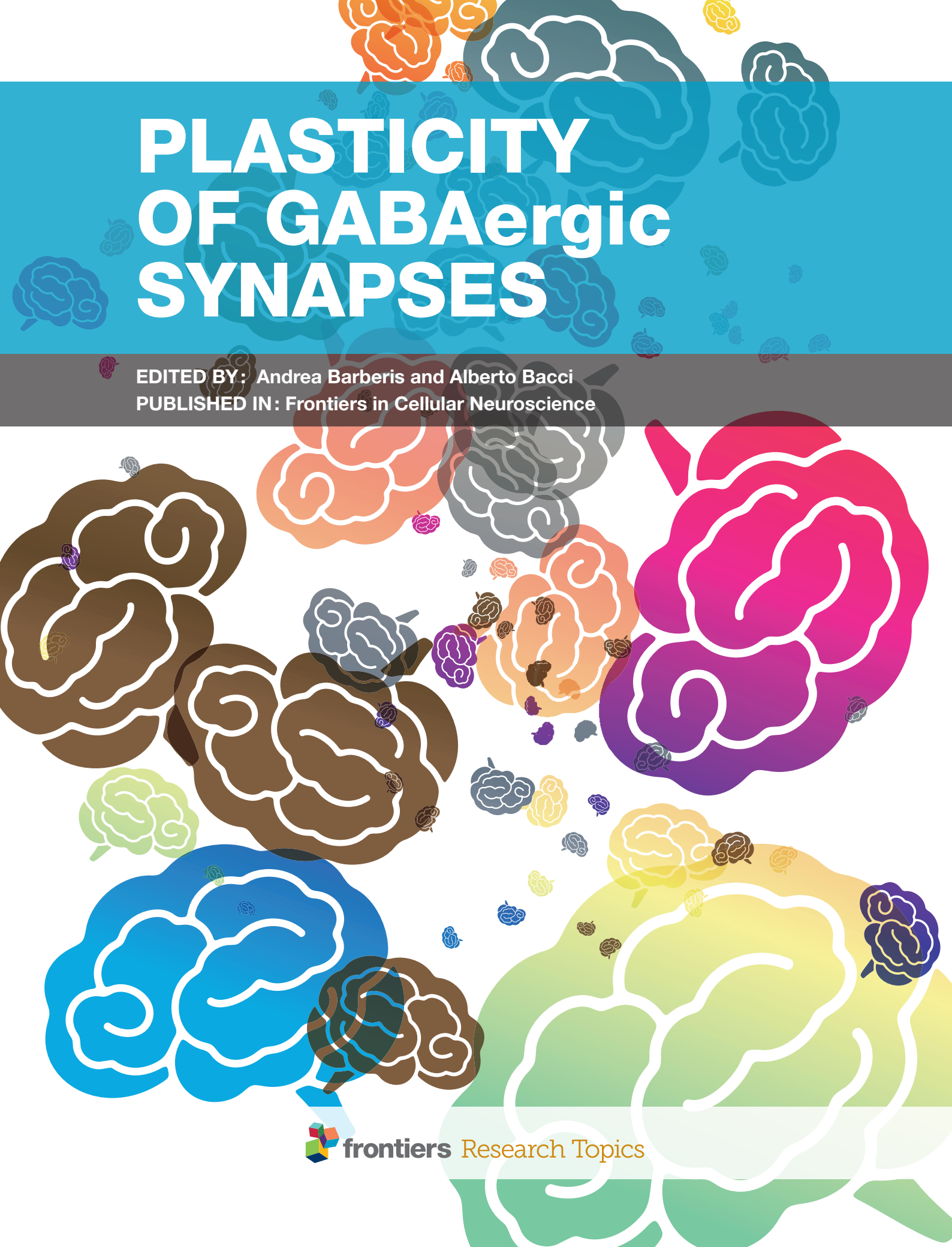
PLASTICITY OF GABAergic SYNAPSES

EDITED BY: Andrea Barberis and Alberto Bacci
PUBLISHED IN: Frontiers in Cellular Neuroscience



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ISSN 1664-8714
ISBN 978-2-88919-732-3
DOI 10.3389/978-2-88919-732-3

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PLASTICITY OF GABAergic SYNAPSES

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Learning and memory are believed to depend on plastic changes of neuronal circuits due to activity-dependent potentiation or depression of specific synapses. During the last two decades, plasticity of brain circuits was hypothesized to mainly rely on the flexibility of glutamatergic excitatory synapses, whereas inhibitory synapses were assumed relatively invariant, to ensure stable and reliable control of the neuronal network. As a consequence, while considerable efforts were made to clarify the main mechanisms underlying plasticity at excitatory synapses, the study of the cellular/molecular mechanisms of inhibitory plasticity has received much less attention. Nevertheless, an increasing body of evidence has revealed that inhibitory synapses undergo several types of plasticity at both pre- and postsynaptic levels. Given the crucial role of inhibitory interneurons in shaping network activities, such as generation of oscillations, selection of cell assemblies and signal integration, modifications of the inhibitory synaptic strength represents an extraordinary source of versatility for the fine control of brain states. This versatility also results from the rich diversity of GABAergic neurons in several brain areas, the specific role played by each inhibitory neuron subtype within a given circuit, and the heterogeneity of the properties and modulation of GABAergic synapses formed by specific interneuron classes.

The molecular mechanisms underlying the potentiation or depression of inhibitory synapses are now beginning to be unraveled. At the presynaptic level, retrograde synaptic signaling was demonstrated to modulate GABA release, whereas postsynaptic forms of plasticity involve changes in the number/gating properties of GABAA receptors and/or shifts of chloride gradients. In addition, recent research indicates that GABAergic tonic inhibition can also be plastic, adding a further level of complexity to the control of the excitatory/inhibitory balance in the brain.

The present Topic will focus on plasticity of GABAergic synapses, with special emphasis on the molecular mechanisms of plasticity induction and/or expression.

Citation: Barberis, A., Bacci, A., eds. (2016). Plasticity of GABAergic Synapses. Lausanne: Frontiers Media. doi: 10.3389/978-2-88919-732-3

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frontiers in Cellular Neuroscience

EDITORIAL
published: 07 July 2015
doi: 10.3389/fncel.2015.00262

Editorial: Plasticity of GABAergic synapses

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Keywords: inhibitory plasticity, iLTP, inhibitory synapse, GABAA receptors, inhibitory microcircuits

For long time, plasticity of brain circuits has been hypothesized to mainly rely on the flexibility of glutamatergic excitatory synapses, whereas inhibitory synapses have been assumed to be relatively invariant. Based on this view, inhibition should be exclusively modulated by the differential glutamatergic-driven activation of a highly diverse population of inhibitory interneurons displaying specific temporal dynamics and selective innervation patterns. However, it has been demonstrated that inhibitory synapses undergo several forms of plasticity, thus providing an additional source of versatility to the regulation of the neuronal network and the emergence of complex brain states.

The cellular and molecular mechanisms occurring at inhibitory synapses during the induction/expression of inhibitory short- and long-term synaptic plasticity are now beginning to be unraveled. At the presynaptic side, retrograde synaptic messengers modulate GABA release (Mendez and Bacci, 2011; Iremonger et al., 2013; Lourenco et al., 2014; Younts and Castillo, 2014), whereas postsynaptic plasticity typically involves changes in the number/gating properties of post-synaptic GABAA receptors (Kurotani et al., 2008; Houston et al., 2009; Luscher et al., 2011; Petrini et al., 2014; Flores et al., 2015). In addition, acute or chronic alterations of intracellular chloride concentration modulate the driving force of GABAergic currents and the subunit composition of GABAA receptors (Woodin et al., 2003; Raimondo et al., 2012; Succol et al., 2012).

The 14 articles presented in this ebook (including hypothesis and theory, minireviews, reviews, and original research articles) cover the mechanisms of inhibitory synaptic plasticity, at the molecular and microcircuit levels. Zacchi et al. (2014) focus on the signaling pathways controlling the phosphorylation state of gephyrin, a key scaffold protein at inhibitory synapses responsible for the synaptic clustering of both glycine and GABAA receptors. By considering the synapse as a highly dynamic element, Petrini and Barberis (2014), review the recent literature addressing the role of protein diffusion in the reorganization of the inhibitory postsynaptic density during inhibitory synaptic plasticity. A similar conceptual approach, based on the analysis of receptor dynamics, has been adopted by Muir and Kittler (2014) to investigate inhibitory plasticity in relation to GABAA receptor diffusion at inhibitory synapses located in the axon initial segment. This original research article reports that chronic depolarization increases the lateral mobility of GABAA receptors and reduces the size of post-synaptic GABAA receptor clusters, thus critically interfering with neuronal excitability. Hirano and Kawaguchi (2014) review another form of postsynaptic inhibitory plasticity observed at cerebellar synapses formed by stellate cells onto Purkinje cells. This inhibitory long-term potentiation involves the CaMKII-dependent increase of GABAA receptor signaling through direct GABAA receptor phosphorylation and/or promoted surface delivery via a GABARAP-dependent mechanism. In their original article, Gao et al. (2014) further address the molecular mechanisms of the aforementioned long-term inhibitory plasticity at cerebellar Purkinje cells. They report that the pathway of iLTP induction critically depends on the coordinated action of both αCaMKII and βCaMKII isoforms, and is modulated by the activation of GABAB receptors.

OPEN ACCESS

Edited and reviewed by:
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Received: *03 June 2015*
Accepted: *23 June 2015*
Published: *07 July 2015*

Citation:
Barberis A and Bacci A (2015) Editorial: Plasticity of GABAergic synapses. Front. Cell. Neurosci. 9:262. doi: 10.3389/fncel.2015.00262

Flores et al. (2015) provide a broad yet detailed analysis of the molecular organization of inhibitory post-synaptic density. In addition, they highlight the formation and elimination of GABAergic synapses as an important source of inhibitory synaptic plasticity. The mini review by Maguire (2014) examines the plasticity of inhibition in response to acute and chronic stress involving region-specific changes of GABAA receptor subunit expression and alterations of the chloride gradient. Moreover, Dr. Maguire reports that stress acts as a metaplastic switch by enabling iLTP at parvocellular neuroendocrine cells (PNCs). Mapelli et al. (2015) provide a comprehensive overview of diverse forms of plasticity at specific cerebellar sub-circuits, introducing the concept of the coordination between excitatory and inhibitory plasticity for correct circuit functioning. In their minireview, Chevaleyre and Piskorowski (2014) highlight the importance of short- and long-term changes of inhibitory synaptic strength in tuning the threshold for the induction of excitatory plasticity. In addition, they discuss how plasticity of glutamatergic synapses onto PV+ interneurons shapes inhibition at hippocampal microcircuits. Pallotto and Deprez (2014) analyze the influence of inhibition in adult neurogenesis in the olfactory bulb and dentate gyrus, by discussing the role of GABAergic signaling in the development and plasticity of adult-born neurons. In their comprehensive review Griffen and Maffei (2014) examine different forms of pre- and post-synaptic inhibitory plasticity occurring at diverse somatosensory cortex interneuron subtypes and discuss the role of such plasticity in sensory cortical activity.

Synaptic signaling does not only depend on pre- or post-synaptic determinants but is also shaped by the dynamics of neurotransmitter in the synaptic cleft. The minireview and the hypothesis and theory by Scimemi (2014a,b) propose the intriguing idea that changes of GABA transporters activity may modulate GABAergic responses. In particular, by exploiting a computer modeling approach, Dr. Scimemi validates the hypothesis that the density, distribution and lateral mobility of GABA transporters affect the GABA concentration sensed by postsynaptic GABAA receptors.

In addition to synaptic inhibition, tonic inhibition produced by the persistent activation of extrasynaptic GABAA receptors is crucial for the tuning of neuronal excitability. Recent evidence demonstrates that also tonic inhibition is plastic. The original article by Barth et al. (2014) illustrates that the ovarian cycle is associated with variations of expression of GABAA receptors containing the "tonic" δ-subunit, both in hippocampal principal cells and interneurons. Interestingly, such plasticity modulates γ-oscillations, thus representing a possible determinant for altered memory and cognitive performance observed during ovarian cycle.

The ability of inhibitory synapses to undergo plasticity emphasized in this ebook raises important questions. First, what are the specific molecular mechanisms of inhibitory plasticity at synapses formed by different interneuron subtypes? Second: how is plasticity orchestrated at both excitatory and inhibitory synapses? In keeping with this, how do different forms of excitatory and inhibitory plasticity co-exist? Do variations of both excitation and inhibition strength occur in parallel/homestatic (Froemke et al., 2007; Xue et al., 2014; Flores et al., 2015), independent (Lourenco et al., 2014), or opposite fashions (Petrini et al., 2014). Are these different "plasticity modes" dependent on the stimulus pattern, specific spatial distributions of synapses and/or time points after plasticity induction? What are the behavioral and cognitive correlates of these different forms of plasticity?

Answering these questions will contribute in redefining the excitation to inhibition balance (E/I) as a "dynamic" activity-dependent determinant for the functioning of brain microcircuits.

Acknowledgments

This work has been supported by Telethon-Italy (GGP11043) and Compagnia di San Paolo (ROL-4318). Investissements d'avenir" ANR-10-IAIHU-06; Agence Nationale de la Recherche (ANR-13-BSV4-0015-01), and a grant from the Institut du Cerveau et de la Moelle épinière (Paris).

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

frontiers in Cellular Neuroscience

REVIEW
published: 05 May 2015
doi: 10.3389/fncel.2015.00169

Integrated plasticity at inhibitory and excitatory synapses in the cerebellar circuit

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The way long-term potentiation (LTP) and depression (LTD) are integrated within the different synapses of brain neuronal circuits is poorly understood. In order to progress beyond the identification of specific molecular mechanisms, a system in which multiple forms of plasticity can be correlated with large-scale neural processing is required. In this paper we take as an example the cerebellar network, in which extensive investigations have revealed LTP and LTD at several excitatory and inhibitory synapses. Cerebellar LTP and LTD occur in all three main cerebellar subcircuits (granular layer, molecular layer, deep cerebellar nuclei) and correspondingly regulate the function of their three main neurons: granule cells (GrCs), Purkinje cells (PCs) and deep cerebellar nuclear (DCN) cells. All these neurons, in addition to be excited, are reached by feed-forward and feed-back inhibitory connections, in which LTP and LTD may either operate synergistically or homeostatically in order to control information flow through the circuit. Although the investigation of individual synaptic plasticities *in vitro* is essential to prove their existence and mechanisms, it is insufficient to generate a coherent view of their impact on network functioning *in vivo*. Recent computational models and cell-specific genetic mutations in mice are shedding light on how plasticity at multiple excitatory and inhibitory synapses might regulate neuronal activities in the cerebellar circuit and contribute to learning and memory and behavioral control.

Keywords: cerebellum, inhibitory synapse, excitatory synapse, LTP, LTD

OPEN ACCESS

Edited by:
Andrea Barberis, Fondazione Istituto Italiano di Tecnologia, Italy

Reviewed by:
Stéphane Dieudonné, Institut de Biologie de l'École Normale Supérieure, France
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Received: *16 February 2015*
Accepted: *16 April 2015*
Published: *05 May 2015*

Citation:
Mapelli L, Pagani M, Garrido JA and D'Angelo E (2015) Integrated plasticity at inhibitory and excitatory synapses in the cerebellar circuit. Front. Cell. Neurosci. 9:169. doi: 10.3389/fncel.2015.00169

Introduction

Various persistent modifications in neuronal and synaptic functioning provide the biological basis of learning and memory in neuronal circuits and, among these, long-term synaptic plasticity (Bliss and Collingridge, 1993) and intrinsic neuronal excitability (Linden, 1999; Hansel et al., 2001; Xu and Kang, 2005) are thought to play a primary role. Long-term synaptic plasticity appears in various forms of potentiation (LTP) and depression (LTD). Although different forms and mechanisms of LTP and LTD have been revealed, often along with forms of intrinsic excitability changes occurring in the same neurons, plasticity in inhibitory subcircuits is still poorly understood. Moreover, the way inhibitory and excitatory mechanisms cooperate in determining brain circuit computations remains unclear. What is most critical is to understand how excitatory and inhibitory plasticity impinging on the same neuron regulate its function, and how excitatory and inhibitory plasticity contribute to microcircuit computation as a whole. This lack of knowledge is somewhat surprising

if one considers that long-term synaptic plasticity is largely believed to play a key role in regulating neuronal and microcircuit operations.

In the cerebellum, long-term synaptic plasticity was initially predicted on theoretical grounds to occur only in the form of LTD or LTP (Marr, 1969; Albus, 1971) at the parallel fiber—Purkinje cell (PF-PC) synapse, but now synaptic plasticity is known to be distributed in the granular layer, molecular layer and deep cerebellar nuclear (DCN; Hansel et al., 2001; Gao et al., 2012) involving both excitatory and inhibitory synaptic transmission as well as neuronal intrinsic excitability. These different forms of plasticity eventually impinge on three main neurons, namely GrCs, Purkinje cells (PCs), and DCN cells, which act therefore as nodes integrating excitatory and inhibitory plasticity (**Figure 1**). Thus, the cerebellum is an ideal system in which the interplay of excitatory and inhibitory plasticity can be investigated. Following their discovery, the possible role of cerebellar plasticities has been hypothesized:

1. Synaptic plasticity in the granular layer may serve to improve spatio-temporal recoding of mossy fiber (MF) inputs into new GrC spike patterns [expansion recoding (D'Angelo and De Zeeuw, 2009)]. Plasticity in the inhibitory Golgi cell (GoC) loop has still to be fully investigated but, based on modeling predictions, it may provide a powerful regulatory mechanism for transmission of appropriate spike trains to PCs.
2. Synaptic plasticity in the molecular layer may serve to store correlated granular layer spike patterns under the teaching signal generated by climbing fibers (CFs) although this latter point is controversial (D'Angelo et al., 2011). This plasticity is in fact composed of multiple mechanisms: different forms of PF-PC LTD and LTP occur together with plasticity in the molecular layer inhibitory interneuron (MLI) network involving GABAergic synapses. For example, PF-PC LTD may occur together with PF-MLI LTP and MLI-PC LTP globally raising PC responses, while PF-PC LTP may occur together with PF-MLI LTD and MLI-PC LTD globally reducing PC responses (Gao et al., 2012).
3. Synaptic plasticity in the DCN may serve to store MF spike patterns (Bagnall and du Lac, 2006; Pugh and Raman, 2006) depending on control signals generated through the cerebellar cortical loop (**Figure 1**). The inhibitory PCs synapses, which regulate DCN activity (Hansel et al., 2001; Boyden et al., 2004; Gao et al., 2012), develop their own LTP and LTD (Morishita and Sastry, 1996; Aizenman et al., 1998; Ouardouz and Sastry, 2000). Recent works (Medina and Mauk, 1999, 2000; Masuda and Amari, 2008) have suggested the importance for MF-DCN and PC-DCN plasticity in controlling cerebellar learning in eye-blink conditioning and vestibulo-ocular reflex (VOR).
4. Long-term changes in intrinsic excitability in GrCs, PCs and DCN cells could further contribute to change the global activity level in these neurons contributing to homeostasis and plasticity (e.g., see Schweighofer et al., 2001).

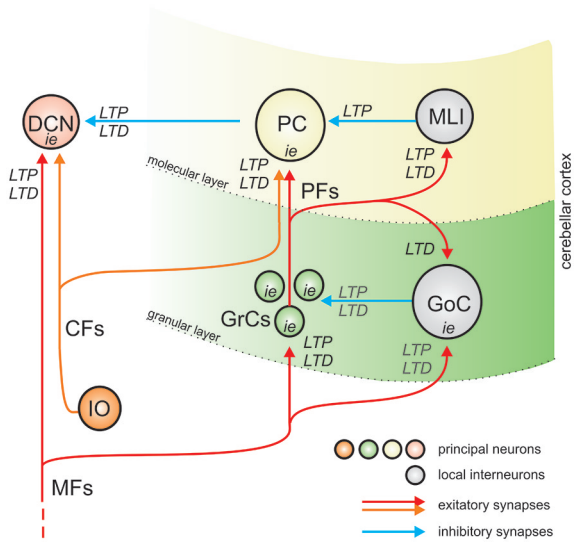

FIGURE 1 | The organization of plasticity in the cerebellar circuit. The drawing shows that the cerebellum is made of three main sub-circuits, comprising the granular layer, molecular layer and deep cerebellar nuclear (DCN). The granular layer and molecular layer form the cerebellar cortex. Both the cerebellar cortex and DCN are activated by MFs, and the cerebellar cortex output inhibits the DCN. Therefore, the cerebellar cortex forms a large inhibitory loop for DCN. Inside cerebellar cortex, in turn, MFs activate GrCs which emit PFs activating Purkinje cells (PCs) and local interneurons inhibit the principal neurons (GoCs inhibit GrCs, MLIs inhibit PCs). PCs and DCN cells are also activated by Climbing fibers (CFs). Therefore, a similar feed-forward inhibitory scheme is implemented in all the three cerebellar subcircuits. *LTP*, *LTD* and plasticity of intrinsic excitability (i.e.,) have been either observed or predicted in all subcircuits. The forms of plasticity determined experimentally are reported in *black*, those predicted by computational modeling are reported in *gray*. Excitatory and inhibitory synapses are represented using *red* and *blue* arrows.

In this review we evaluate the integrated impact of plasticity at inhibitory and excitatory synapses along with long-term changes in intrinsic excitability in the cerebellar circuit and highlight their implications for cerebellar computation.

Long-Term Synaptic Plasticity and Learning in the Cerebellar Circuit

The cerebellum is classically associated with motor control, and learning is thought to subserve the role of calibrating synaptic weights for appropriate response gain regulation and timing. The cerebellum is thought to act through cerebro-cerebellar loops involving the motor cortices (Eccles et al., 1972; Ito, 1972). The critical role in executing precise movements becomes evident when studying patients with cerebellar malfunctioning and diseases, who manifest a sensori-motor syndrome called *ataxia*. Nevertheless, in the last decade a growing body of evidence supported the cerebellar involvement in non-motor, cognitive and emotional functions (Schmahmann, 2010; Schraa-Tam et al., 2012; Voogd, 2012). It is likely that the cerebro-cerebellar loops involved in motor control represent also a model of how the cerebellum takes part in higher functions through reciprocal connections with non-motor brain areas (D'Angelo and Casali, 2012).

The cerebellum controls movements on the millisecond time scale. The motor commands descending from upstream brain areas are relayed to the cerebellum through the pontine nuclei. Once elaborated in the cerebellar circuits, these signals are sent back to the motor cortex through the thalamus to trigger motor acts with appropriate timing (Timmann et al., 1999). The ability to elaborate temporal information on the millisecond time scale led to consider the cerebellum as a *timing* machine (Eccles et al., 1967; Eccles, 1973; Ivry, 1997). As a site of procedural memory, the cerebellum has been predicted to operate as a *learning* machine (Marr, 1969; Ito, 2006). It receives the motor commands from cerebral cortex and, through internal memory of movement inverse dynamics, it is able to elaborate a *prediction* of sensory consequences of motor acts. The sensory prediction is then compared to the sensory feedback to produce a sensory discrepancy signal (Blakemore et al., 2001; Ivry et al., 2002; Ivry and Spencer, 2004). This triad—namely *learning, timing* and *prediction*—emerges as a crucial determinant in adaptive behavior under cerebellar control (De Zeeuw et al., 2011; D'Angelo and Casali, 2012).

Afferent signals are conveyed to the cerebellum through two excitatory pathways composed of the MFs and the CFs. Both these fiber systems send collaterals to the DCN before entering the cerebellar cortex. The MFs contact GrC dendrites in the granular layer of the cerebellar cortex. The GrCs axons generate the PFs, that ascend to the molecular layer and relay the signals onto PCs dendritic arbors. Moreover, PCs directly receive the CFs input and in turn inhibit the DCN. Inhibition in the cerebellar cortex is provided by GoCs interneurons in the granular layer, and stellate (SCs) and basket cells (BCs) in the molecular layer. The DCN cells are inhibited by PCs axons and activated by MF and CF collaterals (Fredette and Mugnaini, 1991; Teune et al., 1995, 2000; Medina et al., 2002). While the PCs represent the only output of the cerebellar cortex, the DCN neurons integrate the PC inhibitory input with the excitatory inputs carried by CFs and MFs collaterals and provide the sole output of the cerebellum.

The regular architecture of the cerebellum has inspired several theories, aiming at understanding how the cerebellum processes incoming information and performs timing and learning functions. According to the *motor-learning theory* (Marr, 1969; Albus, 1971), the property of learning motor skills relies on the cerebellar cortex ability to store stimulus-response associations, by linking inputs with the appropriate motor output. The theory implied that only PF-PC synapses may be modified by experience and that the CF acting as a teacher signal calibrates the PC responsiveness and thus leads the encoding of stimulus-responses associations. The motor-learning theory in the Marr's version implies that, when MFs carry inappropriate information, the PF-PC synapse should be silenced by the olivary input (the opposite would occur according to Albus' version). The hypothetical plasticity of PF synapses postulated by the Motor Learning Theory was observed *in vivo* as a persistent attenuation of PF-PC transmission (PF-PC long term depression, LTD) produced when PF and CF inputs are stimulated together at low frequency (Ito, 1972, 1989). Miles and Lisberger proposed an alternative model (valid at least for the VOR), in which motor learning is achieved through synaptic plasticity at a different site. The instructive signal conveyed by the PC to the vestibular nuclei triggers a change in synaptic efficacy in the connection between MF collaterals and vestibular nuclei (Miles and Lisberger, 1981).

Experimental data provided support for and against each of the two hypotheses, indicating that the explanation of cerebellar motor learning is likely to involve a more complex picture than plasticity at a single synapse. The cellular basis of cerebellar motor learning is generally assumed to be mediated by long-term modifications in the strength of synaptic transmission (for review see Martin et al., 2000). However, the information storage may also involve activity dependent changes in neuronal intrinsic excitability (Armano et al., 2000; Hansel et al., 2001; Zhang and Linden, 2003; Frick and Johnston, 2005; Mozzachiodi and Byrne, 2010).

Different forms of synaptic and non-synaptic plasticity have been described in excitatory and inhibitory neurons of the granular layer, the molecular layer and the DCN (Hansel et al., 2001; Boyden et al., 2004; Gao et al., 2012). Thus, it is likely that cerebellar learning emerges as an integrated process involving various synaptic sites that elaborate, over different time courses, different components of learning (Medina and Mauk, 2000; Medina et al., 2000; van Alphen and De Zeeuw, 2002; Jörntell and Ekerot, 2003; Yang and Lisberger, 2013). However, how remodeling of synaptic weights generates the complex properties of cerebellar learning remains to be understood.

Mathematical models (Mauk and Donegan, 1997; Medina and Mauk, 1999, 2000; Medina et al., 2000, 2001; Ohyama et al., 2003b; Mauk and Buonomano, 2004; Lepora et al., 2010) incorporating more and more details on synaptic connectivity and plasticity at different network sites, may help determining the impact of the different sites of plasticity on cerebellar learning (Shadmehr et al., 2010). These computational approaches have generated several hypotheses, many of which require validation through experimental assessment. Specific tests can be performed either by using mutant mice with alterations in specific plasticity mechanisms (Gao et al., 2012) or by embedding a cerebellar model with multiple learning rules into the control loop of a robotic simulator (Garrido et al., 2013a; Casellato et al., 2014, 2015).

Excitatory and Inhibitory Plasticity in the Granular Layer

In vitro, LTP and LTD at the MF-GrC synapse are associated with changes of GrCs intrinsic excitability (D'Angelo et al., 1999; Armano et al., 2000; Sola et al., 2004; Gall et al., 2005; Nieus et al., 2006; D'Errico et al., 2009). *In vivo*, LTP and LTD can be induced in the granular layer by facial tactile stimulation and intra-cerebellar electrical stimulation (Roggeri et al., 2008). In mathematical models using reconvolution algorithms of granular layer local field potentials, the synaptic and non-synaptic changes reported *in vitro* turned out to be necessary and sufficient to explain those observed *in vivo* (Diwakar et al., 2011). Information on potential changes in the inhibitory circuit are poor at the moment, but they may be synergistic or antagonistic with respect

to those at the MF-GrC relay and regulate information transfer through the granular layer (Arleo et al., 2010; Garrido et al., 2013b).

Plasticity at the MF-GrC Synapse

MF-GrC LTP induction is driven by the coactivation of NMDA receptors (NMDARs) and metabotropic glutamate receptors (mGluRs) (Rossi et al., 1996; D'Angelo et al., 1999; Maffei et al., 2002). The NMDARs are the main source of Ca^{2+} influx that drives synaptic plasticity induction at the MF-GrC relay, while mGluRs represent an amplifying mechanism acting through the IP3 pathway (Finch et al., 1991; Irving et al., 1992a). Voltage-Dependent Calcium Channels (VDCCs) activation, following membrane depolarization and repetitive spike discharges, may also favor MF-GrC LTP (Armano et al., 2000). The intracellular Ca^{2+} signals may be remarkably protracted and amplified by Ca^{2+}-induced Ca^{2+} release (CICR) mechanisms (Irving et al., 1992a,b; Simpson et al., 1996).

Knowing the mechanisms underlying LTP/LTD balance is fundamental to understand how the information is processed and retransmitted by the granular layer. Several intrinsic and extrinsic factors could regulate bidirectional plasticity (for review see D'Angelo, 2014).

First, the patterns of MFs stimulation determine LTP-LTD balance. According to the BCM learning rule (Bienenstock et al., 1982), long-term synaptic plasticity is correlated with the duration of stimulus trains through postsynaptic Ca^{2+} regulation (Gall et al., 2005). Long and repeated MF bursts induce a relatively large Ca^{2+} influx that drives LTP. Instead, LTD is induced by short isolated burst stimulation that causes relatively small Ca^{2+} changes (Gall et al., 2005). Similarly, bidirectional plasticity is influenced by MF stimulation frequencies (D'Errico et al., 2009).

Secondly, nitric oxide (NO) may orchestrate the LTP/LTD balance. High frequency MF stimulation generates a significant NMDAR-dependent and NOS-dependent release of NO in the granular layer (Maffei et al., 2003). As a retrograde messenger, NO regulates the presynaptic release probability, thus driving LTP expression (Maffei et al., 2002). NO release inhibition shifts the balance toward LTD, suggesting that NO is critical for determining plasticity orientation (Maffei et al., 2003).

Thirdly, gating by neuromodulators has been proposed to control LTP and LTD induction at the MF-GrC relay (Schweighofer et al., 2001, 2004). Indeed, it has recently been shown that the cholinergic system enhances MF-GrC LTP through α7-nAChRs activation by shifting the Ca^{2+}-plasticity relationship. In this way, in the presence of nicotine a short MF burst that normally induces MF-GrC LTD, is able to induce LTP, both in acute brain slices and *in vivo* (Prestori et al., 2013). The cholinergic facilitation of LTP induction could be critical for controlling adaptive behaviors like VOR (Schweighofer et al., 2001, 2004; Prestori et al., 2013).

Finally, the combination of synaptic response (excitatory post-synaptic potential, EPSP) and spikes has itself a role in determining plasticity, giving rise to the so-called spike-timing-dependent-plasticity (STDP; Song et al., 2000). Preliminary evidence suggests that STDP could indeed exist at the MF-GrC relay, although the underlying mechanisms remain to be clarified (Sgritta et al., 2014).

Plasticity in the GoC Inhibitory Circuit

Plasticity in the GoC inhibitory circuit may regulate information transfer at the MF-GrC synapse. Following protracted high frequency activation of the MF bundle (typically a theta burst stimulation, TBS) the long-term synaptic plasticity in the granular layer shows a specific spatial organization (Mapelli and D'Angelo, 2007). In particular, LTP and LTD are organized in center-surround structures: more active centers that tend to generate LTP, and less active surrounds that preferentially generate LTD. The sign of plasticity depends on the excitatory/inhibitory balance and therefore it is sensitive to the inhibitory circuit activity. Therefore, GoCs activity may modulate the center-surround organization of signal transmission and bidirectional plasticity at the MF-GrC relay (Mapelli and D'Angelo, 2007; Mapelli et al., 2010, 2014). Although a form of long-term plasticity in the inhibitory GoC-GrC connection has not been described, a recent model suggests that it could represent a potent regulatory mechanism for MF-GrC plasticity (Garrido et al., 2013b). A form of LTD at PF-GoC synapse, following high-frequency burst stimulation of PFs (Robberechts et al., 2010) and long term enhancement of spontaneous GoC firing rate after hyperpolarization (Hull et al., 2013) have been reported (with the first potentially being synergistic and the second homeostatic with respect to the MF-GrC pathway). Also forms of adaptation and long-lasting regulation at the GoC-GrC synapse have been described (Rossi et al., 2006; Mapelli et al., 2009; Brandalise et al., 2012), operating a disinhibition of GrCs in case of high GoCs activation rates, in that being presumably synergistic in our case. However, plasticity at this level remains to be fully investigated. A control of GoC inhibitory activity could also come from GoC-GoC inhibitory synapses (Hull and Regehr, 2012) and gap-junctions (Vervaeke et al., 2010), although the potential impact of these mechanisms on plasticity in the inhibitory circuit is unclear.

Plasticity of GrC Intrinsic Excitability

High frequency activation of the MF-GrC relay (either through a TBS or a high frequency/protracted stimulation) has been shown to induce long-term modifications of GrC intrinsic excitability, along with the LTP of synaptic efficacy. In particular, high frequency stimulation (HFS) is able to determine a long-lasting increase in neuronal responsiveness, increasing the GrC input resistance and reducing the spike threshold. This form of plasticity had been described for the first time in the hippocampus by Bliss and Lomo in 1973 (Bliss and Lomo, 1973). At the MF-GrC synapse, protracted high-frequency stimulations, weaker than the TBS, determine the increase of the intrinsic excitability, leaving unaltered the postsynaptic response amplitude. As for the synaptic LTP, the plasticity of GrC intrinsic excitability is dependent on NMDARs activation (Armano et al., 2000). Indeed, a previous work assessed the role of the NMDA current in enhancing synaptic depolarization and GrC output firing, in particular in response to HFSs (D'Angelo and Rossi, 1998). The mechanism involved in the NMDAR-dependence

probably relies on the calcium ions influx through these receptors (and the consequent activation of calcium-dependent intracellular pathways), rather than on the depolarization consequent to NMDARs opening (Armano et al., 2000). The spike threshold reduction is probably related to a modification of the persistent sodium current and potassium currents (Nieus et al., 2006). The potentiation of GrC responsiveness, and the consequent increase in the number of emitted spikes, reasonably facilitates the development of synaptic LTP. Plasticity of GrC responsiveness could have an additional compensatory role, by restoring granular layer level of excitability in case of weak synaptic excitation (Frégnac, 1998; Armano et al., 2000). Both these mechanisms (synaptic long-term plasticity and plasticity of intrinsic excitability) cooperate in determining the granular layer processing of MF input. Indeed, GrC electrotonic compactness (Silver et al., 1992; D'Angelo et al., 1993) presumably determines that a change in intrinsic excitability would affect neuronal responsiveness as a whole, including synaptic efficacy. Synaptic inhibition mediated by GoCs reasonably affects GrC excitability, through tonic and phasic mechanisms (Armano et al., 2000; D'Angelo et al., 2005). Indeed, GABAergic inhibition modulates GrC excitability in different ways ((Brickley et al., 1996; Rossi et al., 2006); for review (Mapelli et al., 2014)).

Excitatory and Inhibitory Plasticity in the Molecular Layer

The Marr-Albus-Ito hypothesis of cerebellar motor learning implies that the PF input to PCs is the only site of learning in the cerebellar network. However, multiple sites of synaptic plasticity in the molecular layer have been described (Hansel et al., 2001; Boyden et al., 2004; Coesmans et al., 2004; Ito, 2006). The picture emerging from the latest evidences shows that several forms of synaptic plasticity, not only the classical PF-PCs LTD, appear to be involved in cerebellar learning. Here we summarize the principal features of molecular layer plasticity (for a detailed review see Gao et al., 2012; D'Angelo, 2014).

Plasticity at the PF-PC Synapse

Different forms of LTP and LTD, either entirely postsynaptically or presynaptically expressed, have been observed at the PF-PC relay. Thus, four different forms of plasticity may be described: a postsynaptic LTD, a postsynaptic LTP, a presynaptic LTP and a presynaptic LTD.

Postsynaptic PF-PC LTD is induced by paired PF and CF stimulations and involves complex signal transduction pathways. The activation of AMPARs and mGluRs following PF stimulations induces, through different mechanisms, a postsynaptic Ca^{2+} transient that, over a certain threshold, may activate protein kinase C (PKC; Hartell, 2002). Active PKC phosphorylates the AMPARs at the PC terminals and drives their desensitization and internalization, resulting in LTD of PF-PC relays (Wang and Linden, 2000; Xia et al., 2000). CF stimulations contribute to generate large widespread Ca^{2+} transients, through AMPARs, NMDARs, and VGCCs activation (Konnerth et al., 1992; Piochon et al., 2010). In particular, postsynaptic CF-PC NMDARs are necessary for LTD (but not for LTP) at the PF-PC synapse, when PF activation is paired with CF activation (Piochon et al., 2010). CaMKIV (Boyden et al., 2006) and α/βCaMKII (Hansel et al., 2006; van Woerden et al., 2009) are necessary both for PF-PC LTD and for motor learning. Nevertheless, this form of LTD does not strictly require CF activity and may be induced through PF stimulation alone (Ohtsuki et al., 2009). Other mechanisms could amplify local Ca^{2+} signals and allow LTD induction, as somatic depolarization (Linden et al., 1991) or strong PFs activation (Hartell, 1996; Eilers et al., 1997). Indeed, the simultaneous activation of several PFs stimulated at 1 Hz, at relatively high stimulus intensity, may generate postsynaptic Ca^{2+} transients that remain confined to spines (Midtgaard et al., 1993; Denk et al., 1995), reaching the levels for the LTD induction (Hartell, 1996). However, when the CFs are stimulated a lower stimulus strength is sufficient for LTD induction (Han et al., 2007). Therefore, although it is clear that CF activity facilitates PF-PC LTD, CF involvement is not strictly required (Ohtsuki et al., 2009). Additionally, intense PF stimulations (as brief burst of 2–5 pulses at 10–50 Hz for 1–2 min) may induce heterosynaptic LTD (Marcaggi and Attwell, 2007), in which the LTD may spread to PF synapses tens of microns away from the original site, through second messengers such as NO (Reynolds and Hartell, 2000; Wang et al., 2000) and arachidonic acid (Reynolds and Hartell, 2001). The NO pathway is necessary for the heterosynaptic LTD induction (Crepel and Jaillard, 1990; Shibuki and Okada, 1991; Daniel et al., 1993) and provides another important mechanism involved in plasticity induction at the PF-PC synapses. Indeed, the NO produced by PFs (Southam et al., 1992; Kimura et al., 1998) or by MLIs (Carter and Regehr, 2000) activates a NO-dependent form of guanylate cyclase (GC) in PCs, thus activating the cGMP/PKG pathway, whose effect is to prevent the dephosphorilation of AMPARs by blocking the PP2/PP1/PP2B cascade and therefore unblocking PKC (Lev-Ram et al., 1995, 1997; Linden et al., 1995; Gao et al., 2012).

Postsynaptic PF-PC LTP is induced by single pulses PF stimulation at 1 Hz for 5 min, driving GluR2 AMPARs subunit insertion in the spine membrane, through a mechanism dependent on the activation of the PKA, PKC and CAMKII pathways (Lev-Ram et al., 2002; Coesmans et al., 2004; Belmeguenai and Hansel, 2005; Kakegawa and Yuzaki, 2005; van Woerden et al., 2009). The use of selective mutant mice can help investigating the mechanisms underlying these forms of plasticity and their role *in vivo*. In particular, the L7-PP2B mice, in which the PP2B was deleted only in cerebellar PCs, allowed to determine that this molecule is necessary for PF-PC LTP and for correct VOR and eye-blink conditioning (Schonewille et al., 2010). Postsynaptic LTP and LTD are mutually reversible, modulating the AMPARs desensitization and membrane expression (Lev-Ram et al., 2003; Coesmans et al., 2004). The sign of plasticity is determined by several factors, depending on the different induction mechanisms, the NO pathway and the postsynaptic Ca^{2+} transients. In general, stimulation patterns that generate a relatively low Ca^{2+} influx drive LTP while relatively high Ca^{2+} transients are associated with LTD (Coesmans et al., 2004). This is an opposite scenario of that predicted by the "BCM rule", in which lower and

higher Ca^{2+} transients are associated with the induction of LTD and LTP respectively (Bienenstock et al., 1982). This property of PF-PC plasticity may have profound impact on cerebellar information processing.

Presynaptic PF-PC LTP may be induced by low-frequency (2–8 Hz) PF stimulations (Sakurai, 1987; Crepel and Jaillard, 1990; Hirano, 1991; Shibuki and Okada, 1992), determining an increase of presynaptic Ca^{2+} influx that activates the adenyl cyclase (AC1) pathway. The consequent activation of PKA determines the phophorilation of the vesicle-release related proteins thus increasing neurotransmitter release (Salin et al., 1996; Kimura et al., 1998; Storm et al., 1998; Powell et al., 2004). In addition, NO released by neighboring synapses may regulate the probability of glutamate release and LTP induction in non-activated PF terminals (Hartell, 2002; Qiu and Knöpfel, 2007; Le Guen and De Zeeuw, 2010). On the contrary, the endocannabinoids released after a high frequency bursts, suppress in PF terminals the AC1 pathway, activating the cannabinoid 1 (CB1) receptors, thereby preventing the induction of presynaptic LTP (van Beugen et al., 2006). A cannabinoid-mediated affect at this level has been described as consequent of the activation of the cholinergic system, mediated by muscarinic receptors (Rinaldo and Hansel, 2013).

Presynaptic PF-PC LTD also requires the activation of CB1 receptors. This LTD may emerge after a low-frequency stimulation when presynaptic LTP is pharmacologically prevented, providing a mechanism of bidirectional plasticity at the presynaptic site (Qiu and Knöpfel, 2007).

In conclusion, the cholinergic system and endocannabinoid receptors are able to deeply modulate synaptic plasticity at the PF-PC connection, at various levels. Notably, these two system proved able to interact (Rinaldo and Hansel, 2013). As it is true also for other cerebellar regions, cholinergic activation is able to modulate synaptic activity and plasticity, also mediating the release of other neurotransmitters, therefore influencing local neuronal activity (Turner et al., 2011).

Plasticity at the CF-PC Synapse

The CFs activity may play an important role in regulation of LTP/LTD balance at the PF-PC synapses. The CF stimulation facilitates postsynaptic PF-PC LTD induction by enhancing dendritic Ca^{2+} signals and by releasing the neuropeptide CRF. Moreover, CF activity triggers the release of endocannabinoids from PC dendrites and suppresses the presynaptic PF-PC LTP (Ohtsuki et al., 2009). The high probability of neurotransmitter release at the CF terminals (Dittman and Regehr, 1998) as well as the all-or-none character of CF signaling (Eccles et al., 1966) has induced to consider the CF-PC synapses as "unmodified" synapses. However, low-frequency (5 Hz, 30 s) CF stimulation may induce LTD of PC responses (Hansel and Linden, 2000; Carta et al., 2006). The CF-LTD is postsynaptically induced and expressed (Shen et al., 2002) and it is associated with an alteration in the complex spike waveform (Hansel and Linden, 2000), a reduction in the complex spike afterhyperpolarization (Schmolesky et al., 2005), and a depression of CF-evoked dendritic Ca^{2+} transient (Weber et al., 2003). The CF-LTD has a significant effect on the probability of induction of postsynaptic LTD and LTP at PF-PC synapses (Coesmans et al., 2004). The reduction in complex spike-associated Ca^{2+} transients following the CF-LTD is sufficiently strong to reverse the polarity of postsynaptic plasticity at the PF-PC relay. Indeed, when CF-LTD is induced first, subsequent application of PF-PC LTD induction protocol results in LTP (Coesmans et al., 2004). A form of CF-PC LTP has been described during development in mice (around 4–11 postnatal days) (Bosman et al., 2008; Ohtsuki and Hirano, 2008). This LTP requires large CF inputs and is dependent on postsynaptic Ca^{2+} increase although being independent on NMDARs activation (Bosman et al., 2008). Since CF innervations on PC shows a 1:1 ratio in adult animals (while more CF impinge on the same PC during development), the CF-PC LTP observed in newborn mice could help strengthen one CF connection, while determining the pruning of the others (Bosman et al., 2008; Ohtsuki and Hirano, 2008).

Plasticity in the MLI Inhibitory Circuit

PF-MLI synapses and MLI-PC synapses are both sites of plasticity. Different forms of long-term plasticity have been described in PF-MLI relays: a postsynaptic LTD, a postsynaptic LTP and a presynaptic LTP.

Postsynaptic PF-MLI LTD can be induced by sustained PFs stimulations (repeated sequences of 4 × 25 stimuli at 30 Hz) and requires the activation of Ca^{2+}-permeable AMPARs, mGlur1Rs and CB1Rs. The postsynaptic Ca^{2+} influx that drives LTD induction is confined at activated synapses (Soler-Llavina and Sabatini, 2006). Moreover, this synapse-specific plasticity drives the membrane expression of GluR2-containg Ca^{2+}-impermeable AMPARs, thus providing a self-limiting mechanism (Liu and Cull-Candy, 2000; Sun and June Liu, 2007). PFs stimulation paired with SCs depolarization, which could follow CFs activations (Szapiro and Barbour, 2007), can induce a postsynaptic PF-MLI LTP (Rancillac and Crépel, 2004). This LTP depends on NO and/or cAMP (Rancillac and Crépel, 2004). *In vivo* PF-MLI LTP may be induced by simultaneous activation of PFs and CFs inputs, resulting in long-lasting increases in receptive fields of MLIs (Jörntell and Ekerot, 2002).

Presynaptic LTP at PF-MLI synapses has been described after PF stimulations (at 8 Hz for 30 s (Bender et al., 2009)) and provides a positive feedback mechanism. Indeed, GABA released from MLI diffuses in the extracellular space and activates GABAARs at nearby PF terminals. GABAARs activation leads to an increase in PF release probability and an increase of the excitability of the axon and soma/initial segment, potentiating synaptic transmission onto MLI (Pugh and Jahr, 2011).

CF activation can induce a long-lasting potentiation of PCs spontaneous and evoked inhibitory post-synaptic currents (IPSCs), a phenomenon that is called *rebound potentiation* (Kano et al., 1992). This form of MLI-PCs LTP requires the increase of intracellular Ca^{2+} concentration and is caused by the upregulation of GABAAR activity on PCs (Kano et al., 1996; Hashimoto et al., 2001; Kawaguchi and Hirano, 2007).

There are therefore several mechanisms that could come into play to make synaptic plasticity in the MLI inhibitory circuit either synergistic or antagonistic with respect to plastic changes occurring at the PF-PC synapse.

Plasticity of PC Intrinsic Excitability

PC excitability may be enhanced by somatic current injection or the PFs stimulation protocols that induce PF-LTP (Belmeguenai et al., 2010). The PCs intrinsic plasticity shares with LTP the activation of protein phosphatises 1, 2A and 2B for the induction (Belmeguenai et al., 2010). It also requires PKA and casein kinase 2 (CK2) activity and it is mediated by the downregulation of different K^+ channel-mediated conductances, such as A-type K^+ channels and probably Ca^{2+}-activated K^+ currents (Schreurs et al., 1998). PC intrinsic plasticity, resulting in enhanced spine Ca^{2+} signaling, lowers the probability of subsequent LTP induction. Thus, intrinsic PC plasticity follows LTP of active PF synapses and reduces the probability of subsequent LTP at weaker, non-potentiated synapses.

Excitatory and Inhibitory Plasticity in DCN

The DCN (as well as the vestibular nuclei, VN) are strategically located within the cerebellar circuitry, in a position ideal to integrate the information coming from brain stem, inferior olive (IO) and spinal cord with the PCs output coming from the cerebellar cortical loop, and provide the sole output of the cerebellum. Experimental investigations using pharmacological tools and focal lesions have revealed that the DCN play an important role in associative learning, such as in eyelid conditioning or VOR adaptation (Lavond et al., 1985; Steinmetz et al., 1992). Evidence that these forms of cerebellar motor learning induce plasticity in DCN and VN (Lisberger, 1994; du Lac et al., 1995; Kleim et al., 2002; Ohyama et al., 2006) suggested that memory storage was not limited to the cerebellar cortex. Long-term modifications in synaptic strength have been described in the inhibitory synapses between PCs and DCN neurons and in the excitatory synapses between MFs and DCN. In addition, persistent changes of the intrinsic excitability have been observed in DCN neurons. Recently, it has been suggested that PC-DCN and MF-DCN synapses are plastic on a slow time scale and store persistent memory. Conversely, plasticity in cerebellar cortex could operate on a shorter time scale, storing transient memory that could then be transferred downstream and consolidated through DCN plasticity in slow phases of learning (Medina and Mauk, 1999; Medina et al., 2000; Masuda and Amari, 2008).

Plasticity at the MF-DCN Synapse (Excitatory)

DCN neurons show a robust post-inhibitory rebound spike burst after stimulation of inhibitory PCs synapses (Gardette et al., 1985a,b; Aizenman and Linden, 1999). This rebound hallmark induced by PC activity drives the plastic changes of the MF-DCN glutamatergic synapse. A MFs high-frequency burst that precedes a DCN post-inhibitory rebound depolarization induces a synapse-specific MF-DCN LTP (Pugh and Raman, 2006). This LTP induction protocol mimics the predicted time course of excitation and inhibition during delay eyelid conditioning. The MFs convey the conditioned stimulus, while the unconditioned stimulus is carried by the CFs. The DCN neurons receive excitation directly from the MFs collaterals, followed by the indirect inhibition via the GrC-PC-DCN circuit (Mauk et al., 1986; Hesslow et al., 1999). The PCs respond to the unconditioned stimulus with a complex spike, followed by a brief pause that allows the post-inhibitory firing in the nuclei. During cerebellar learning of associative tasks, the acquisition of the conditioned response depends on the correct timing between the MF-mediated excitation and the PC-mediated inhibition that drives excitatory post-synaptic currents (EPSCs) potentiation (Ohyama et al., 2003a). The PCs fire as a response to MF activation, but when the conditioned and unconditioned stimuli are paired and repeated, PCs firing slows down during the final phase of the conditioned stimulus. This would generate a disinhibition in DCN neurons, allowing the generation of the excitatory response that elicits a blink (Jirenhed et al., 2007). LTP cannot be induced when the timing of synaptic excitation and hyperpolarization is modified. With longer intervals between excitation and inhibition, or with a reverse sequence, EPSCs tend to depress (Pugh and Raman, 2008).

MF-DCN LTP depends on both NMDAR and low-voltage-activated Ca^{2+} channels, activated respectively by synaptic excitation and inhibition (Pugh and Raman, 2006). DCN are spontaneously active neurons and express NR2D subunit-containing NMDARs, generating channels weakly blocked by Mg^{2+} (Akazawa et al., 1994). Therefore, unlike other brain regions, the MF-DCN LTP is not consequent to the coincidence detection of signals that generates a suprathreshold increase in the intracellular Ca^{2+} level. MF-DCN plasticity rather depends on the timing of two different signals that act independently to activate distinct intracellular signaling pathways. This mechanism may be adequate to encode temporal information that is required for non-Hebbian and adaptive plasticity during associative learning tasks (Medina and Mauk, 1999; Pugh and Raman, 2009). The excitation drives the Ca^{2+} influx in individual synapses, with NMDARs providing the priming signal. The inhibition generates a global signal that triggers LTP induction only in the primed synapses (Pugh and Raman, 2008). Multiple signaling cascades may be activated by priming and trigger signals. The Ca^{2+} influx through NMDARs activates the calcium-dependent phosphatase calcineurin, while the Ca^{2+} influx through the high-voltage-activated Ca^{2+} channels activates the calmodulin-dependent protein kinase II (CaMKKII). At the same time, the potentiation of the primed synapse is triggered only if the suppressive effect of L-type Ca^{2+} current is reduced by hyperpolarization (Person and Raman, 2010). This provides evidence that synaptic inhibition plays an active role in the induction of MF-DCN LTP.

Moreover, a form of MF-DCN LTD has been reported, which can be induced by MFs high-frequency burst stimulation, either alone or paired with postsynaptic depolarization. Again, a postsynaptic Ca^{2+} transient is needed to the induction of this plasticity, that it is blocked by Ca^{2+} chelators (Zhang and Linden, 2006). MF-DCN LTD is NMDAR independent and requires the activation of the group I metabotropic glutamate receptor 1 (mGluR1) and protein translation (Zhang and Linden, 2006).

Plasticity at the PC-DCN Synapse (Inhibitory)

LTP of IPSCs in DCN neurons can be induced after HFS at 100 Hz (Ouardouz and Sastry, 2000) of PC axons, while stimulation at lower frequencies (as 10 Hz), induces LTD (Morishita and Sastry, 1996). The PC-DCN tetanus-induced long-term plasticity does not require GABARs activation (Morishita and Sastry, 1996; Ouardouz and Sastry, 2000). LTP and LTD appear to depend on NMDAR activation and on intracellular Ca^{2+} increase, as they are both blocked by NMDAR antagonist APV and/or the calcium chelator BAPTA. Moreover, depolarizing pulses that activate VGCCs in DCN neurons induce LTP when given at 2 Hz or LTD when given at 0.1 Hz (Morishita and Sastry, 1996; Aizenman et al., 1998; Ouardouz and Sastry, 2000). Therefore, a large Ca^{2+} influx through NMDAR or L-Type Ca^{2+} channels drives LTP of IPSCs, while LTD is induced by moderate Ca^{2+} increases. The plasticity induced by depolarization pulses is weaker than that induced by tetanus (HFS). The Ca^{2+} increase that follows the depolarizing pulses driving a smaller LTP, remains mainly located in the DCN soma and proximal dendrites (Muri and Knöpfel, 1994; Aizenman et al., 1998). In contrast, HFS may act also on CFs and MFs collaterals, whose excitatory synapses are distributed on DCN soma as well as on proximal and distal dendrites (Ikeda and Matsushita, 1973, 1974). The consequent NMDARs activation leads to a larger increase of intracellular calcium, both in the soma and in the dendritic tree, inducing a stronger IPSCs LTP.

PCs activity drives the plasticity of the inhibitory synapses in DCN neurons, but the sign of the bidirectional plasticity strikingly depends on excitatory synapses activation level. Therefore, the activation of MF or CF collaterals influences the induction of LTP (Ouardouz and Sastry, 2000) or LTD (Morishita and Sastry, 1996), by regulating the Ca^{2+} influx through the NMDARs.

Plasticity of DCN Intrinsic Excitability

High-frequency MF bursts induce potentiation of intrinsic excitability in DCN neurons (Aizenman and Linden, 2000; Zhang et al., 2004). After the EPSP bursts, the input resistance and the number of action potentials evoked by a depolarization pulse increase, while the spike threshold decreases. Also the rebound depolarization that follows a hyperpolarization step is increased. All these mechanisms enhance DCN neurons excitability. Similar to the GrC intrinsic excitability increase following MF-GrC LTP (Armano et al., 2000), the changes in DCN intrinsic excitability depend on NMDARs activation and require an increase in intracellular Ca^{2+} concentration. MF stimulation may also drive LTP in DCN (Pugh and Raman, 2006), suggesting that potentiation of intrinsic excitability coexists with potentiation of synaptic efficacy, again similar to MF-GrCs LTP. The increase in neuronal excitability amplifies synaptic potentiation, enhancing the ability of DCN neurons to respond to MF inputs (Zheng and Raman, 2010). Also PC inhibitory post-synaptic potential (IPSP) bursts can induce a persistent and Ca^{2+}-dependent increase of DCN intrinsic excitability (Zhang et al., 2004). The changes in DCN neurons excitability caused by PC firing increase followed by a brief pause, might play an important role in motor learning tasks (Zhang et al., 2004).

Coordination of Multiple Forms of Excitatory and Inhibitory Plasticity

As described above, the three main cerebellar subcircuits (granular layer, molecular layer and DCN) are all sites of complex forms of plasticity, some occurring at excitatory and some at inhibitory synapses (D'Angelo, 2014). However, despite numerous hypotheses have been formulated, the main question remains: how does plasticity at excitatory and inhibitory synapses interact in controlling cerebellar circuit functioning? There are four general considerations that need to be taken into account in order to answer the questions.

First, the granular and molecular layer subcircuits share a similar inhibitory architecture, with a feed-forward inhibitory loop passing through the local inhibitory interneurons and controlling retransmission through the primary neuron (in addition, the granular layer also has a feed-back inhibitory loop). Moreover, the whole cerebellar cortex acts as a third large feed-forward inhibitory loop controlling retransmission through the DCN (**Figure 1**).

Secondly, synaptic plasticity is present at both excitatory and inhibitory synapses, distributed at several connections at the granular layer, molecular layer and DCN. These different forms of synaptic plasticity are expected to develop in a coordinated manner following signal inputs to the cerebellum.

Thirdly, in each subcircuit, inhibitory interneurons fine-tune the principal neuron output and the critical issue is whether inhibitory plasticity tends to compensate and rebalance changes (*homeostatic effect*) or rather reinforces and amplifies the effects of excitatory plasticity occurring in the main neuronal pathway (*synergistic effect*).

Finally, plasticity is probably dynamically transferred through the cerebellar circuit synapses into deep structures and possibly also outside the cerebellum, e.g., in the cerebral cortex and brainstem (Koch et al., 2008). Cerebellar plasticity seems therefore unavoidably bound to local circuit dynamics (D'Angelo and De Zeeuw, 2009) and to the extended recurrent networks formed by the cerebellum with extracerebellar areas.

Insight from Experimental Recordings

In order to provide a key to interpret the role of the various plastic mechanisms reported in the cerebellar circuit (see above), it would be helpful to develop a plausible hypothesis of excitatory and inhibitory plasticity interaction using a prototypical demonstration. It is already known that a sensory stimulus like the TSS (theta-sensory stimulus) delivered to the rat whisker pad is able to induce LTD of granular layer response to MF input *in vivo* (Roggeri et al., 2008). Since this effect is expected on the basis of MF-GrC plasticity rules *in vitro* (Armano et al., 2000; Sola et al., 2004; D'Errico et al., 2009), it can be hypothesized that synaptic plasticity at the inhibitory GoC connections does not counterbalance MF-GrC LTD. Therefore, plasticity in the Golgi cell loop may be synergistic with that developed at the MF-GrC relay and may effectively increase GrC inhibition. As a consequence, PFs would convey a decreased level of excitation to the molecular layer. Given the inverse "BCM rule" at the PF-PC connection,

a weak PFs activation pattern could lead to PF-PC LTP, as suggested by preliminary data (Ramakrishnan and D'Angelo, 2012). Similarly to the granular layer, the inhibitory feed-forward loop in the molecular layer (PF-MLI-PC) could act synergistically with the PF-PC synapse through the induction of PF-MLI LTD, further boosting PC responses. Little is known about the CFs activation following the TSS, although it is likely that it would considerably affect PF-PC behavior. The consequent increase in PC responsiveness could lead to LTP at the PC-DCN connection, increasing PC inhibition of DCN cells. This, in turn, would favor the onset of post-inhibitory rebound depolarization colliding with MFs high-frequency burst activity conveyed by MFs. Since MF activity precedes DCN post-inhibitory rebound depolarization, MF-DCN LTP would be favored (Pugh and Raman, 2006). This example shows a concatenation of events providing a plausible hypothesis of how excitatory and inhibitory plasticity could act synergistically to modify MF input processing and integration through the cerebellar cortex and DCN. This picture, although deliberately oversimplified, provides a working hypothesis on the events that might develop in cerebellar cortex following patterned inputs on the afferent MF pathway. Clearly, introducing CF inputs and their potential instructive role on PF-PC plasticity is another primary factor that should be considered to reshape the landscape of plasticity and signal transmission through the cerebellar network.

Insight from Cerebellar Network Models

Cerebellar modeling has traditionally focused on the classical Marr-Albus' hypothesis of cerebellar learning (Marr, 1969; Albus, 1971), accounting for plasticity only at the PF-PC connection. According to the Marr-Albus' hypothesis, the cerebellum operates like a perceptron (Albus, 1971). The PF-PC synapses adapt their weights depending on CF activity (assumed to carry error-related signals) and GrC activity (assumed to perform expansion recoding of sensory inputs reaching the cerebellum through the MFs). Although the Marr-Albus' hypothesis does not account for the numerous forms of cerebellar plasticity and totally ignored any potential role for inhibitory plasticity, it has inspired most cerebellar models elaborated so far. Surprisingly, in these models based on the Marr-Albus' hypothesis, the cerebellum succeeded in solving different kinds of tasks, including eyelid conditioning (Medina and Mauk, 2000), VOR adaptation (Masuda and Amari, 2008) and object manipulation (Luque et al., 2011) or even multiple tasks together demonstrating generalization (Casellato et al., 2014). The fact is that learning in these tasks was oversimplified and far from biological realism, so that these models provided a proof of principle that the cerebellum requires plasticity to perform sensory-motor control rather than explaining how its internal plasticity mechanisms operate.

Plasticity in the granular layer has long been neglected in computational models. The GrCs have been supposed to sparsify the MFs incoming signals based on a combinatorial principle (Yamazaki and Tanaka, 2007), exploiting their huge number and connectivity. However, some models have proposed that MF-GrCs and GoC-GrC plasticity may improve granular layer sparse coding of MF inputs (Coenen et al., 2001; Schweighofer et al., 2001; Philipona and Coenen, 2004; Rössert et al., 2014). According to these models, Hebbian learning in the MF-GrC synapses, operating in conjunction with anti-Hebbian learning in the GoC-GrC synapses and homeostatic intrinsic plasticity in both GrCs and GoCs, maximizes the information transfer between the MFs and the GrCs, generating a sparse representation of the MF input. Recent models suggested how cerebellar granular-layer coding could take advantage of spike-timing and distributed plasticity (Garrido et al., 2013b; Rössert et al., 2014). Variations in multiple weights distributed among different connections succeeded to regulate the number of GrC spikes and their positioning with millisecond precision in response to MF bursts. The weight at MF-GrC synapses (main transmission pathway) effectively controlled the first-spike delay, as previously shown experimentally (Arleo et al., 2010). Modeling of weight changes at the inhibitory GoC-GrC together with the excitatory MF-GrC connections revealed the key role of inhibition in shaping the timing and precision of GrC firing (Nieus et al., 2014). The weight at MF-GoC synapses (feed-forward inhibitory loop) and PF-GoC synapses (feed-back inhibitory loop) regulated the duration of the excitatory time-window during which the first spike could be emitted. Moreover, the weights at the GoC-GrC synapses (common inhibitory loop) and GoC-GoC (inhibitory interneuron network) weights controlled the intensity and duration of GrC inhibition and the number of emitted spikes. Therefore, plasticity in the inhibitory circuit of the granular layer could effectively shape the spatio-temporal time-windows of PF discharge.

Distributed plasticity, depending on different combinations of weights at excitatory and inhibitory synapses, proved able to change information flow through the main MF-GrC excitatory pathway favoring different aspects of network processing in turn (**Figure 2**): (i) increasing transmission (when inhibition on GrCs is depressed); (ii) filtering (in case of inhibition increase and simultaneous LTD at the MF-GrC relay); (iii) maximize time precision (when LTP prevails at all connections in the subcircuit); and (iv) maximize bursting (when inhibition is depressed while the MF-GrC relay potentiates). This model is of particular relevance, since it defines the different functional states that could be achieved by the granular layer circuit in different phases of the learning process. While increasing transmission may be useful to enable the learning process, maximizing timing, filtering or bursting could be the end-point of a specific circuit learning process (Garrido et al., 2013b).

Other models have long hypothesized the role of plasticity in DCN afferent synapses (Medina et al., 2001; Masuda and Amari, 2008; Garrido et al., 2013a; Clopath et al., 2014). These models generally agree that MF-DCN plasticity consolidates the information that has previously been acquired due to the molecular layer plasticity. The separation of learning in two stages (fast learning and consolidation) has been shown to enhance the learning capabilities in eyeblink conditioning (Medina et al., 2001; Monaco et al., 2014), VOR (Masuda and Amari, 2008; Clopath et al., 2014) and complex manipulation tasks (Garrido et al., 2013a). However, this last model has proposed that distributed plasticity in the DCN (including both

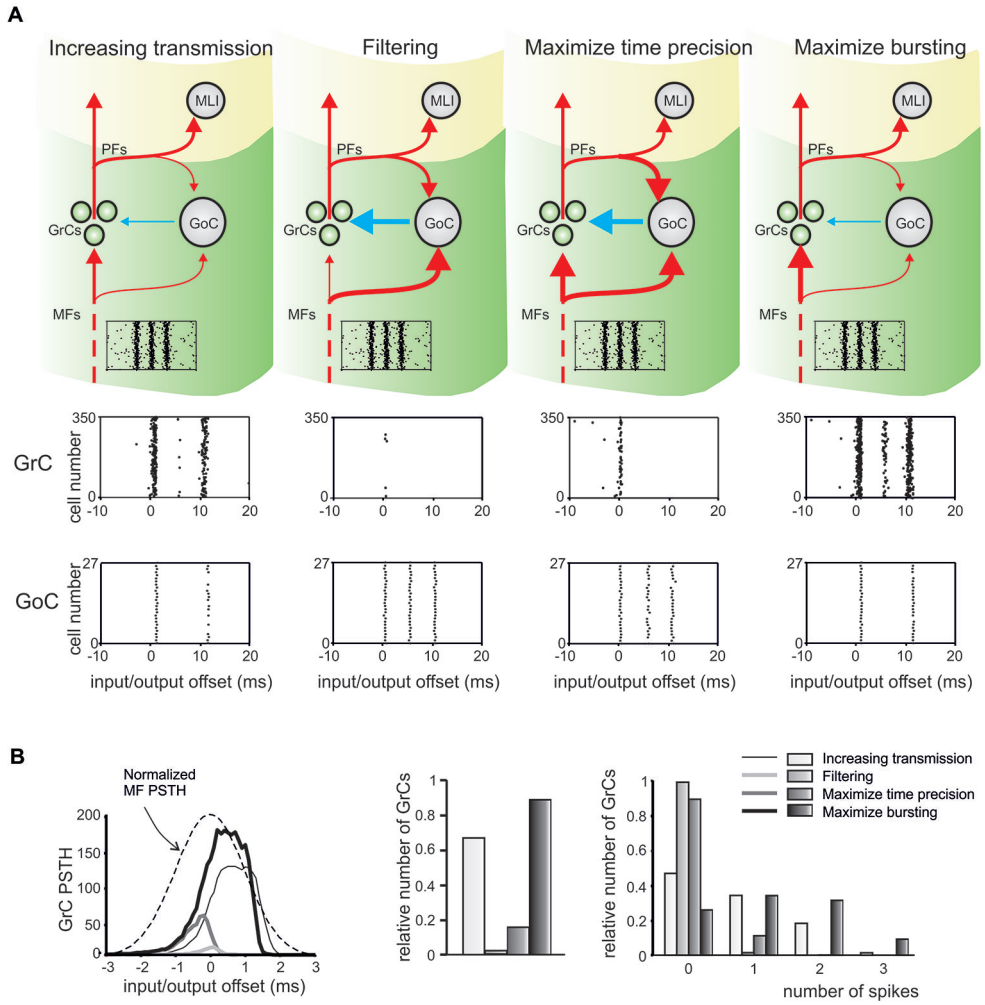

FIGURE 2 | Integrated regulation of microcircuit functions by synaptic plasticity at excitatory and inhibitory synapses. This figure shows the effect of results of integrated regulation of microcircuit functions by synaptic plasticity at excitatory and inhibitory synapses in a computational model of the cerebellar granular layer. **(A)** The line thickness in the circuit schemes illustrates the relative synaptic weights for the four different conditions (same colors and circuit elements as in **Figure 1**) and the raster plots indicate the Mossy fiber (MF) input. Systematic changes in synaptic weights could generate four different effects: (1) *increase transmission*; (2) *signal filtering*; (3) *maximize time precision*; and (4) *maximize bursting*. The GrC and GoC firing in response to the MF input burst are shown in the raster plots for each condition. **(B)** The peri-stimulus time histograms (PSTH) show the relative number of GrCs generating spikes in response to the input. The PSTHs change in the four different conditions. The nature of changes is illustrated in the histograms, showing the relative number of GrCs responding to the input pattern with 0, 1, 2, or 3 spikes. *Modified from Garrido et al. (2013b)*.

the MF-DCN and PC-DCN plasticity) could also store gain information, keeping the PCs operating in their optimal firing range and avoiding their saturation. According to this model, while the PF-PC synapses stored information related with the correlation between sensorial state representations (along the sparse GrC activity) and the associated error in the task under development (represented in the CF activity), the DCN afferents could store information about the gain of the task, enhancing the generalization capabilities of the cerebellum. The IO-DCN connection was not considered until a recent computational model proposed that it could act as an internal feed-back loop (Luque et al., 2013), accelerating the convergence of learning without conflicting with the generalization capabilities previously suggested to the MF-DCN and PC-DCN synapses. Moreover, the hypothesized existence of short-term plasticity in that connection could effectively enable/disable this feedback loop based on the error evolution.

These computational models are therefore providing new hypotheses on how inhibitory and excitatory plasticity could integrate to generate the cerebellar output. What is most interesting is that, in *closed-loop robotic simulations*, the multiple forms of long-term synaptic plasticity can effectively enable adaptive motor control with properties—*prediction, timing and learning* (Ivry, 1997; Ivry et al., 2002; Shadmehr et al., 2010)—and temporal dynamics similar to those observed in humans (Casellato et al., 2014, 2015; Luque et al., 2014). The main inhibitory plasticity in these neurorobots was located in the PC-DCN synapse (**Figure 3**). In these robotic tests, the

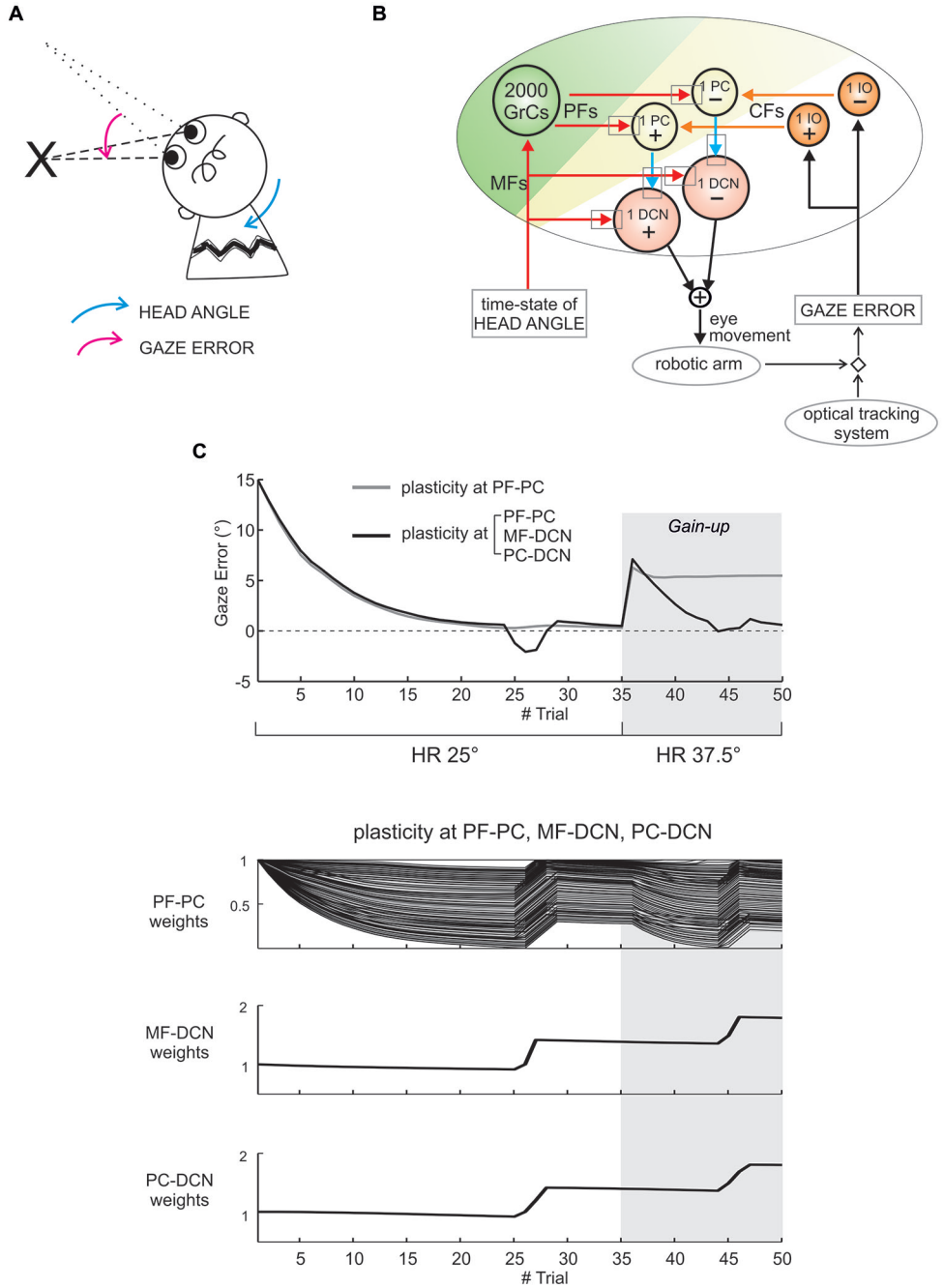

FIGURE 3 | Distributed cerebellar plasticity in real-robot sensorimotor vestibulo-ocular reflex (VOR) task. (A) Human-like VOR task: the arrows show the head angle rotation (*blue arrow*) and the angle of gaze error (*pink arrow*). **(B)** Cerebellar model with VOR-specific input and output signals. The plasticity sites are indicated by the *gray rectangles* (all plasticities are bidirectional with LTP and LTD rules defined and calibrated according to experimental observations, see (Casellato et al., 2014, 2015; Luque et al., 2014)). *Red and orange arrows* indicate excitatory connections from mossy fibers (MFs) and CFs, respectively. *Blue arrows* indicate inhibitory connections. The head vestibular stimulus represents the system time-state, decoded by the granular layer. The gaze error is fed into the CF pathway, and the DCN neurons modulate compensatory eye movements. **(C)** Gain-up VOR test: after 35 trials, the head rotation (HR) was increased 1.5 times (from 25° to 37.5°), and imposed for other 15 trials. The curves report the gaze error within each of the total 50 trials, implementing plasticity at a single site (PF-PC connection, *in gray*) or at multiple sites (PF-PC, MF-DCN and PC-DCN connections, *in black*). With one or three plasticities, the robot compensated equally well for HR. However, while plasticity at the PF-PC connection alone proved unable to change the gain and to correct for the increased HR, combined plasticities at PF-PC, MF-DCN and PC-DCN were able to rescale the response and adapt to the new HR angle. The three bottom plots show synaptic weights at the end of each trial for the three synapses involved, referring to the case of plasticity at the three connections. Indeed, the transfer from cortical to nuclear sites made the PF-PC synapses ready for further plasticity, making them able to react to perturbations suddenly presented to the system. Modified from Casellato et al. (2015).

PF-PC synapse could rapidly learn the contextual information needed to compensate for movement errors. With a slower kinetics, the PC-DCN and MF-DCN synapses were able to store this information in a stable form leaving the PF-PC synapse capable of readapting rapidly. This gave the system a remarkable flexibility preventing PF-PC synaptic weight saturation and allowing its reuse in different tasks, for example when the same muscle district was engaged in different motor tasks and when signal rescaling was needed. Therefore, distributed plasticity seems essential to endow the neuronal circuit with biologically effective properties. Moreover, plasticity at the inhibitory PC-DCN synapse appears to be critical to effectively tune transmission along the large side-loop formed by the cerebellar cortex onto DCN. In future works, more realistic representations of neuronal circuits and learning rules will have to be included into closed-loop robotic systems in order to improve our understanding on how integrated plasticity at inhibitory and excitatory synapses controls functioning of the cerebellar circuit.

Conclusions

This revisitation of cerebellar network plasticity shows that LTP and LTD at inhibitory synapses, and more in general in the inhibitory loops, are needed to fine tune the activity at crucial neuronal nodes located along the main circuit transmission pathway. The combination of synaptic weight change at excitatory and inhibitory synapses can effectively shape the network activity states. These states could change dynamically in order to enable different phases of the learning process and transfer of plasticity inside and outside the local circuit. Experimental and modeling evidence suggests that, in certain conditions, plasticity at inhibitory and excitatory synapses could have synergistic effects. Therefore, introduction of inhibitory plasticity allows to draw a new picture of cerebellar circuit functioning beyond the original intuition that learning has just to occur through plasticity at the PF-PC synapse. There is now the need for several critical demonstrations to fully understand the integrated role of inhibitory and excitatory plasticity in the cerebellar circuit. First, learning rules at inhibitory and excitatory synapses and their interdependence need to be determined experimentally. This investigation has to take into account the modulatory states and input patterns relevant to control plasticity and may make use of mutant mice with alteration in specific synaptic mechanisms. Secondly, the effective occurrence of LTP and LTD *in vivo* in response to specific stimuli or learning protocols needs to be clarified. Thirdly, network models incorporating realistic learning rules need to be extended in order to simulate plasticity dynamics in the circuit. Finally, closed-loop robotic simulations are needed to determine the effective engagement of network learning mechanisms during complex tasks. In this framework, the cerebellar network is likely to provide a very effective workbench.

Acknowledgments

This work was supported by European Union grants to ED [CEREBNET FP7-ITN238686, REALNET FP7-ICT270434, Human Brain Project (HBP-604102)] and by Centro Fermi grant [13(14)] to LM.

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

frontiers in
CELLULAR NEUROSCIENCE

REVIEW ARTICLE
published: 27 October 2014
doi: 10.3389/fncel.2014.00327

Shaping inhibition: activity dependent structural plasticity of GABAergic synapses

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Inhibitory transmission through the neurotransmitter γ-aminobutyric acid (GABA) shapes network activity in the mammalian cerebral cortex by filtering synaptic incoming information and dictating the activity of principal cells. The incredibly diverse population of cortical neurons that use GABA as neurotransmitter shows an equally diverse range of mechanisms that regulate changes in the strength of GABAergic synaptic transmission and allow them to dynamically follow and command the activity of neuronal ensembles. Similarly to glutamatergic synaptic transmission, activity-dependent functional changes in inhibitory neurotransmission are accompanied by alterations in GABAergic synapse structure that range from morphological reorganization of postsynaptic density to de novo formation and elimination of inhibitory contacts. Here we review several aspects of structural plasticity of inhibitory synapses, including its induction by different forms of neuronal activity, behavioral and sensory experience and the molecular mechanisms and signaling pathways involved. We discuss the functional consequences of GABAergic synapse structural plasticity for information processing and memory formation in view of the heterogenous nature of the structural plasticity phenomena affecting inhibitory synapses impinging on somatic and dendritic compartments of cortical and hippocampal neurons.

Keywords: GABAergic synapses, structural plasticity, activity-dependent plasticity, interneuron, memory, cerebral cortex, hippocampus

INTRODUCTION: GABAERGIC SYSTEM AS A SUBSTRATE FOR BRAIN PLASTICITY

During the last decades neuroscientists and physiologists worldwide have made an herculean effort to elucidate the mechanisms of activity-driven changes in synaptic strength and understand its physiological significance as the brain substrate for learning and memory (Malenka and Bear, 2004; Mayford et al., 2012). Recent observations have added an additional level of complexity to activity-dependent synaptic plasticity of excitatory synaptic transmission by showing that adaptive changes in glutamatergic synapse strength are accompanied by a dynamic regulation of excitatory synapse structure (Yuste and Bonhoeffer, 2001; Matsuzaki et al., 2004). Activity-driven structural changes include actin-dependent enlargement of the postsynaptic density (Matus, 2000; Honkura et al., 2008) and formation and elimination of excitatory synapses (Holtmaat and Svoboda, 2009). *In vivo* studies have shown that dendritic spines, the morphological correlate of glutamatergic synapses in excitatory neurons, are formed and eliminated in response to synaptic activity patterns induced by learning behavior (Xu et al., 2009) and that formation of durable memories is directly correlated with the stability and formation of new excitatory synapses (Yang et al., 2009). In addition to functional plasticity, the structural rearrangement of glutamatergic synapses is critically involved in the brain processes leading to learning and memory formation (Caroni et al., 2012).

The plasticity of inhibitory neurotransmission has received relatively less attention than its excitatory counterpart despite its potential to deeply alter the function of cortical networks. A recent attempt to unravel different forms of plasticity in inhibitory γ-aminobutiric acid (GABA) releasing neurons has proven to be unexpectedly successful and cover many different aspects of the physiological properties of inhibitory cell's, including glutamatergic inputs, dendritic and axonal structure, passive properties and GABAergic synapses onto target cells (Kullmann et al., 2012). As glutamatergic contacts, inhibitory synapses have the remarkable property of being able to alter the efficiency of synaptic transmission according to the patterns of activity that flow through them. Research carried out during the last two decades has made clear that inhibitory synapses undergo short- and long-term forms of plasticity and numerous examples of activity dependent changes in synaptic strength of GABAergic synapses have been described in different brain areas, including hippocampus and cortex (Gaiarsa et al., 2002; Castillo et al., 2011; Méndez and Bacci, 2011). The complex and varied collection of pre- and postsynaptic mechanisms that underlie the induction and expression of GABAergic synapse plasticity mirrors the heterogeneous nature of different inhibitory neuron subtypes

that form cortical GABAergic synapses (Méndez and Bacci, 2011).

It is now clear that, similar to excitatory synapses, the molecular composition and structure of GABAergic synapses show a high degree of dynamism (Kittler and Moss, 2003; Lévi et al., 2008; Chen et al., 2012; van Versendaal et al., 2012). The changing nature of inhibitory synapse structure raises the possibility that functional alterations in inhibitory neurotransmission may occur through structural rearrangements. Indeed, synaptic activity driven functional changes of inhibitory neurotransmission are accompanied by modifications in the structure of GABAergic synapses with two major consequences: alteration of synaptic size and morphology and formation and elimination of inhibitory contacts (**Figure 1**). Here we review several aspects of the structural plasticity of mammalian cortical and hippocampal GABAergic synapses. What is the nature of the morphological changes affecting hippocampal and cortical inhibitory synapses? What is the role of synaptic activity and experience in the induction of structural alterations of inhibitory synapses and what molecular mechanisms underlie this form of plasticity? In view of the staggering diversity of different inhibitory neuron subtypes that configure cortical networks, what are the potential physiological consequences of the structural remodeling of GABAergic synapses? We attempt to answer these questions with the aim of providing a framework to understand the characteristics, mechanisms and functions of inhibitory synapse structural remodeling.

GABAERGIC SYNAPSES AND FUNCTIONAL PLASTICITY

Inhibitory synapses, also known as symmetrical synapses (or type II) by their ultrastructural features (**Figure 2A**; Gray, 1959; Colonnier, 1968) are arranged around the scaffold protein gephyrin, the main molecular organizer of inhibitory synapses (Sassoè-Pognetto et al., 2011; Tyagarajan and Fritschy, 2014). Gephyrin forms submembranous hexagonal macromolecular complexes (Xiang et al., 2001; Fritschy et al., 2008) that orchestrate multiple protein-protein interactions with $GABA_A$ Receptors ($GABA_A$ R), the cytoskeleton and various cell adhesion and signal transduction proteins (**Figure 2B**).

Inhibitory synapses are non-uniformly distributed along the different subcellular compartments of pyramidal cells. In the hippocampus, the highest density of GABAergic inputs is found in the soma and proximal dendrites compared to intermediate and distal dendrites (Megías et al., 2001). GABAergic synapses are formed by a highly heterogeneous group of cells (Markram et al., 2004; Ascoli et al., 2008) that mostly correspond to the definition of interneuron (locally projecting neuron with axonal arborization, dendritic and somatic compartments in the same anatomic structure). Interestingly, some interneurons (INs) form synapses

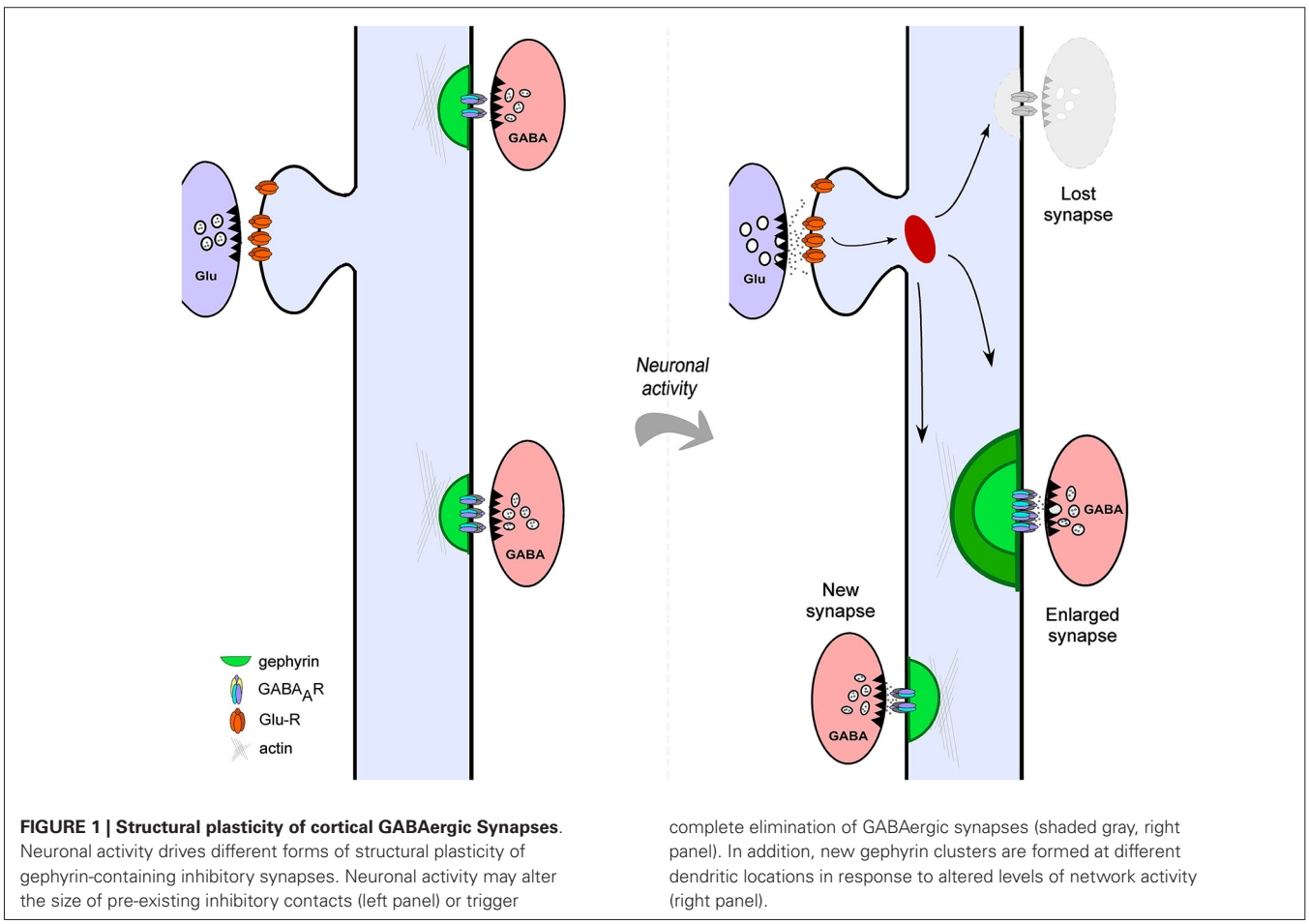

FIGURE 1 | Structural plasticity of cortical GABAergic Synapses. Neuronal activity drives different forms of structural plasticity of gephyrin-containing inhibitory synapses. Neuronal activity may alter the size of pre-existing inhibitory contacts (left panel) or trigger complete elimination of GABAergic synapses (shaded gray, right panel). In addition, new gephyrin clusters are formed at different dendritic locations in response to altered levels of network activity (right panel).

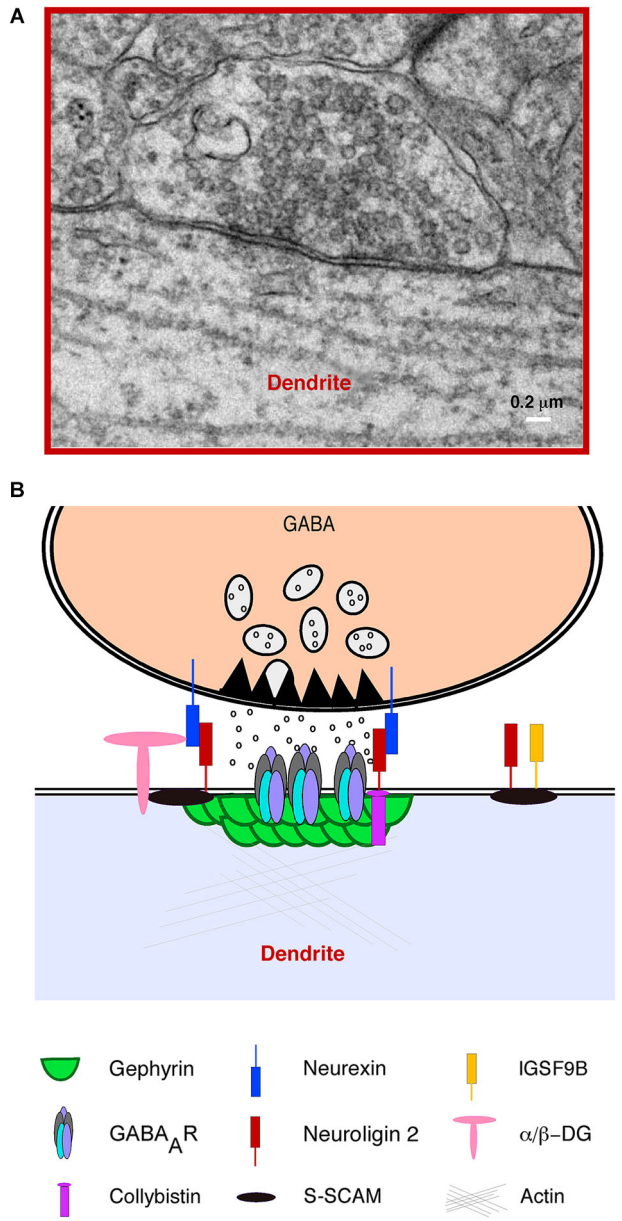

FIGURE 2 | Structural features and molecular composition of an hippocampal GABAergic Synapse. (A) Electron microscopy (EM) image of an inhibitory (symmetrical synapse) between a GABAergic presynaptic terminal and a proximal apical dendrite of a CA1 hippocampal pyramidal neuron. The image shows typical ultrastructural features of inhibitory synapses: a distinguishable synaptic cleft, pleomorphic GABA containing vesicles and a thin post synaptic density (PSD) facing an active zone of similar width. **(B)** A simplified cartoon of a typical GABAergic synapse illustrating the presynaptic terminal with vesicles containing GABA, the active zone, presynaptic neurexins and the inhibitory postsynaptic density showing a vast number of postsynaptic proteins including $GABA_A$ receptors and the scaffold protein gephyrin.

exclusively on the dendrites of other neurons (Maccaferri, 2005), while other target the somatic compartment (Freund and Katona, 2007). This highly stereotyped axonal targeting of GABAergic synapses to specific cellular compartments allows different INs subtypes to selectively affect the different computational processes that actively integrate synaptic inputs in the soma and dendrites (Miles et al., 1996; Pouille and Scanziani, 2001, 2004). Adult INs have a critical role in maintaining physiological activity levels, stabilizing neuronal networks and preventing runaway excitation through different forms of GABA mediated inhibition. In addition, INs are able to form synaptic contacts with a large number of neighboring neurons and provide synchronous inhibition to functionally significant portions of the network (McBain and Fisahn, 2001). In this way, INs coordinate the spiking activity of large number of cells and are critically involved in the genesis of the wide variety of rhythmic network activities that are the basis of cognition and behavior (Klausberger and Somogyi, 2008; Buzsáki and Wang, 2012).

FUNCTIONAL PLASTICITY OF CORTICAL GABAERGIC SYNAPSES

The heterogeneous population of cortical INs shows an equally diverse range of mechanisms of activity driven changes in synaptic strength of GABAergic neurotransmission. Retrograde signaling has been shown to play a prominent role in the modulation of GABAergic synaptic plasticity. Activity-dependent synthesis and release of different signaling molecules by post-synaptic excitatory neurons induce short and long-term forms of plasticity in cortical inhibitory synapses. Endocannabinoids are synthesized by postsynaptic neurons in response to increased Ca^{2+} concentrations, action potential trains and metabotropic glutamate, dopamine, and acetylcholine receptor activation (Kano et al., 2009). Endocannabinoids travel retrogradely and activate CB1 receptor, a G-protein coupled receptor located mainly on presynaptic terminals, resulting in short or long term decreases in GABA release (Piomelli, 2003; Chevaleyre et al., 2006). Another form of desinhibition by retrograde signaling is mediated by postsynaptic somatodendritic glutamate release and activation of presynaptic metabotropic glutamate receptors (Zilberter, 2000). In addition, several examples of spike-timing dependent plasticity of GABAergic synapses have been described in the cortex and hippocampus. This form of plasticity requires near coincident pre- and postsynaptic spiking. The precise rules that dictate the sign of plasticity, potentiation or depression of GABAergic synapses seem to differ between hippocampus (Woodin et al., 2003), entorhinal cortex (Haas et al., 2006) and neocortex (Holmgren and Zilberter, 2001). High frequency stimulation of synaptic inputs has also the potential to produce long term depression (LTD) and potentiation of GABAergic neurotransmission (Komatsu, 1996; Komatsu and Yoshimura, 2000; Patenaude et al., 2003) by pre- (Chevaleyre and Castillo, 2003) and postsynaptic mechanisms (Lu et al., 2000). Astrocyte-dependent Ca^{2+} signaling (Kang et al., 1998) has been shown to modulate the strength of GABA synaptic signaling in the hippocampus. In the neocortex layer 4 excitatory neurons coupling of postsynaptic subthreshold depolarizations with presynaptic action potentials of presynaptic INs triggers a form of long term potentiation (LTP) of GABAergic synaptic transmission that is modulated by sensory activity (Maffei et al., 2006).

All these forms of induction and expression of functional plasticity at inhibitory GABAergic synapses occur through a large variety of mechanisms. Changes in GABAergic synaptic strength

are mediated by mechanisms that range from post-transcriptional modifications of GABA receptors such as phosphorylation (Vithlani and Moss, 2009), ubiquitination (Saliba et al., 2007), trafficking (Vithlani et al., 2011) and lateral diffusion (Lévi et al., 2008; Bannai et al., 2009), to alterations in presynaptic GABA release and variations in chloride (the main $GABA_A$ receptor permeable ion) driving force (Woodin et al., 2003) and give rise to short and long terms changes in inhibitory synapse efficacy. Other forms of plasticity of inhibitory transmission depend on GABA and glutamate postsynaptic signaling through metabotropic and ionotropic receptors (Lu et al., 2000; Wang and Maffei, 2014). Although during the last decades our knowledge of the modes and mechanisms of activity dependent changes in GABAergic synaptic strength has grown enormously (Kullmann et al., 2012), we have a limited knowledge of the structural remodeling that accompanies most of these functional changes. Structural remodeling of GABAergic synapses may represent an essential mechanism for activity dependent regulation of GABAergic function.

STRUCTURAL PLASTICITY OF GABAERGIC SYNAPSES

Examples of activity mediated structural plasticity of GABAergic synapses have been observed during the development of the mammalian brain. Brain patterning occurs through genetic programs that guide the generation and migration of INs and the innervation patterns, geometry and target specificity of GABAergic axonal projections (Hébert and Fishell, 2008; Bartolini et al., 2013). In mice, maturation of GABAergic connectivity occurs both embryonically and during the first postnatal weeks, leaving open the possibility of an experience dependent modulation of inhibitory synapse formation. Indeed, during early stages of brain formation, experience and activity dependent processes overlap with genetically encoded mechanisms of development and regulate several aspects of GABAergic synaptogenesis, including axonal branching, formation of GABAergic synaptic contacts and synaptic strength (Huang et al., 1999; Doischer et al., 2008; Huang, 2009). It has been shown that reduced synaptic activity induced by sensory deprivation in young, but not adult animals, produces specific reductions in the number of perisomatic inhibitory synapses on cortical excitatory cells, unmasking a regulatory role of neuronal activity in determining the density of inhibitory cell contacts in this specific cellular compartment (Jiao et al., 2006). Other studies have provided similar results using pharmacological and genetic manipulation of activity in developing hippocampal cultures (Marty et al., 2000; Hartman et al., 2006). Interestingly, these studies point to a homeostatic role of inhibitory synapse structural plasticity in compensating alterations in global activity levels of developing cortical and hippocampal networks. GABA content in INs is a critical mediator of GABAergic innervation in the developing visual cortex. Knocking down Glutamic Acid Decarboxilase 67 (GAD67), the main GABA synthesizing enzyme in cortical INs, resulted in serious deficits in axonal branching and decreased formation and size of perisomatic synapses on cortical pyramidal neurons (Chattopadhyaya et al., 2004, 2007). The regulation of GAD67 expression and function by synaptic activity suggests that GABA itself could be a mediator of activity dependent structural remodeling of GABAergic synapses.

ACTIVITY DEPENDENT ULTRASTRUCTURAL CHANGES IN GABAERGIC SYNAPSES

Subtle activity induced changes in adult GABA synapse morphology have been studied using electron microscopy (EM), which allows the unequivocal identification of symmetrical GABAergic synapses and the analysis of synaptic ultrastructure at very high resolution. This technique has shown that patterns of activity that produce functional and structural changes in excitatory synapses also induce structural remodeling of inhibitory synapses. Both *in vitro* (Lushnikova et al., 2011) and *in vivo* (Nusser et al., 1998), the rise in synaptic activity levels increased inhibitory synaptic junctional area and complexity and proportion of somatic cell surface covered with inhibitory postsynaptic densitiy (PSD). In other cases, different aspects of inhibitory synapse plasticity occur in a coordinated manner. Bourne and Harris (2011) used LTP inducing protocols and EM three dimensional reconstructions of CA1 pyramidal neurons dendritic segments in acute hippocampal slices to show that plasticity inducing protocols produce a decrease in dendritic inhibitory PSD density that is counterbalanced by an extension of the individual PSD areas. These experiments demonstrate that the adult inhibitory PSD is endowed with mechanisms that allow dynamic changes in the structure in response to alterations in the levels of network activity.

Chronic sensory deprivation by whisker trimming induces a net decrease in the number of symmetric GABAergic synapses in the dendrites of layer 4 neurons of barrel cortex, the main target neurons for the thalamocortical axons relaying sensory information from the whiskers (Micheva and Beaulieu, 1995). Artificially increasing single whisker activity by passive stimulation leads to a rise in dendritic inhibitory synapse density in the correspondent barrel but not in the neighboring ones (Knott et al., 2002). Interestingly, sensory deprivation (through whisker trimming) and stimulation (by artificial chronic movement of the whiskers) result in opposite effects on the number of dendritic inhibitory synapses by preferentially affecting inhibitory synapses contacting dendritic spines and, to a much lower extent, those formed in the shaft of dendrites of principal cells (Micheva and Beaulieu, 1995; Knott et al., 2002). Inhibitory synapses of the barrel cortex are also remodeled in response to learning. A conditioning paradigm involving a group of whiskers induces an increase in the number of symmetric synapses and GABA content of inhibitory axons impinging on dendrites of layer 4 pyramids of the barrel corresponding to the trained whiskers (Jasinska et al., 2010). As in the cases described above, this de novo, learning induced GABAergic synaptogenesis affected inhibitory contacts on dendritic spines but not those on the dendritic shafts of layer 4 excitatory neurons. It is clear that a direct positive relation exists between synaptic activity and GABA synapse formation in the adult somatosensory cortex and that spine GABA synapses, represent a highly structurally dynamic pool of synapses.

INHIBITORY AXON PLASTICITY

A major difficulty in the study of inhibitory synapse dynamics is the lack of morphological markers at the optical level. However, different approaches have been used to track cortical GABAergic structural plasticity. Transgenic mouse lines with genetically labeled subpopulation of INs allow the visualization of inhibitory

axon dynamics in organotypic hippocampal slice cultures and in the cortex *in vivo*. A pioneer study gained insight in the mechanisms of formation of new inhibitory synapses on CA1 pyramidal cell dendrites *in vitro* using high resolution fluorescence confocal imaging of the sites of contact of inhibitory axons and postsynaptic structures (Wierenga et al., 2008). Although the vast majority of putative contact sites were stable, the authors were able to detect formation of new inhibitory contacts between GABAergic axons and dendrites of excitatory neurons. Interestingly, new stable contacts were formed at location were pre- and postsynaptic structures were in close apposition (Wierenga et al., 2008). Close observation of the presynaptic component alone has shown that the majority of putative presynaptic structures is stable and shows abundant expression of inhibitory pre and post synaptic proteins (Schuemann et al., 2013). Short-lived boutons show however, lower levels of GABA synapse markers (Schuemann et al., 2013), suggesting a protracted maturation of new GABAergic contacts. Interestingly, the level of network activity directly controlls inhibitory axon plasticity and produces subtle but significant changes in bouton turnover and morphology (Schuemann et al., 2013). Tracking GABAergic axons dynamics *in vivo* has been possible by implanting cranial widows in mice expressing a fluorescent protein in a subpopulation of inhibitory neurons and visualizing inhibitory presynaptic structures using confocal microscopy in superficial cortical layers (Keck et al., 2011). A certain degree of inhibitory axons structural remodeling has been observed even in conditions of normal sensory activity in the somatosensory cortex: while the length of axonal projections from inhibitory cells in the barrel cortex remains constant, boutons are added and eliminated at a rate of 10% per week (Marik et al., 2010). However, under conditions of altered sensory input by whisker removal, axons from inhibitory neurons in the deprived barrels suffer intense structural modifications, retracting terminals in the vicinity of their cell body and extending collaterals beyond its normal projection range towards non-deprived barrels two days after sensory deprivation. In addition, whisker trimming produces at the same time a general decrease in bouton density (Marik et al., 2010). Similar manipulation of sensory inputs in the visual cortex, synaptic input deprivation by permanent lesion of the retina, induces the disappearance of a fraction of GABAergic boutons in few hours (Keck et al., 2011), suggesting that the rapid loss of functional inhibitory synapses may represent a general adaptive mechanism to decreased levels of synaptic activity that is conserved in different functional areas of the cortex. Axons of GABAergic neurons are dynamic structures, able to alter structural properties in response to altered levels of synaptic activity and sensory experience. By growing and retracting axons, INs are able to increase or decrease the number and change the identity of their postsynaptic targets. The appearance and elimination of boutons suggest the involvement of a mechanism that coordinates changes in pre- and postsynaptic structures during structural plasticity of GABAergic synapses.

THE POSTSYNAPTIC SIDE PLASTICITY

Although tracking presynaptic structures identifies changes in putative inhibitory synaptic contacts the fluorescent tagging of the scaffolding protein gephyrin allows dynamic visualization of the postsynaptic component of GABAergic synapses (**Figure 3**). During developmental inhibitory synaptogenesis, gephyrin accumulates progressively at sites of new synapse formation following a similar pattern as presynaptic components such as the vesicular GABA transporter VGAT, (Dobie and Craig, 2011). Developing inhibitory postsynapses show a high degree of structural plasticity including translational movements along dendrites in a coordinated manner with presynaptic axons and trafficking of synaptic vesicles from pre-existing boutons to new ones (Dobie and Craig, 2011). However, synaptic activity drives the maturation of inhibitory neurotransmission and results in stable and functionally stronger GABAergic synapses compared with those observed during the early phases of inhibitory synaptogenesis (Dobie and Craig, 2011; Vlachos et al., 2013).

The use of postsynaptic tagging of inhibitory synapses has the advantage of resolving the subcellular compartments where GABAergic axons impinge on postsynaptic neurons. Using this approach, two recent studies (Chen et al., 2012; van Versendaal et al., 2012) have revealed important features of structural plasticity of GABAergic synapses in visual cortical circuits *in vivo*. Under normal conditions, a continuos turnover affects a low proportion of GABAergic synapses in dendrites of layer 2/3 pyramidal neurons of the visual cortex. New GABA synapses replace lost inhibitory contacts and tend to be persistent in the majority of the cases (Chen et al., 2012), which contrasts with the low stability of new excitatory connections observed in young and adult animals (Holtmaat et al., 2005). GABAergic synapses on apical dendrites of layer 2/3 pyramidal neurons are preferentially removed in response to decreased sensory inputs by visual deprivation. Interestingly, not all GABAergic synapses on dendrites of pyramidal cells seem to be equally plastic. The most dynamic population of GABA synapses are those contacting dendritic spines (specially abundant in distal parts of layer 2/3 cortical pyramidal neurons) compared with inhibitory synapses made on the dendritic shafts (Chen et al., 2012; van Versendaal et al., 2012). Inhibitory synapses are thus dynamic structures formed and eliminated in cortical neurons *in vivo* in response to physiological changes in network activity levels. Under these same conditions, no alteration in density or turnover of excitatory connections is observed in the same cells (Hofer et al., 2009), suggesting that structural plasticity of GABA synapses and subsequent rewiring of inhibitory microcircuits is a fundamental mechanism for brain adaptation to sensory experience in layer 2/3 pyramidal cells.

MOLECULAR MECHANISMS OF ACTIVITY-DEPENDENT STRUCTURAL PLASTICITY OF GABAERGIC SYNAPSES

The experiments discussed above clearly show that network activity is a major driving force for structural remodeling of inhibitory synapses. Central to the molecular machinery that links synaptic activity with such plastic changes is the Neuronal Per Arnt Sim domain protein 4 (NPAS4), a brain-specific basic helix-loop-helix transcription factor, whose expression is tightly regulated by synaptic activity and postsynaptic calcium influx (Flavell and Greenberg, 2008). Neuronal Per Arnt Sim domain protein 4 regulates the formation of somatic and dendritic inhibitory synapses during development with little effect on excitatory contacts (Lin et al., 2008). In addition,

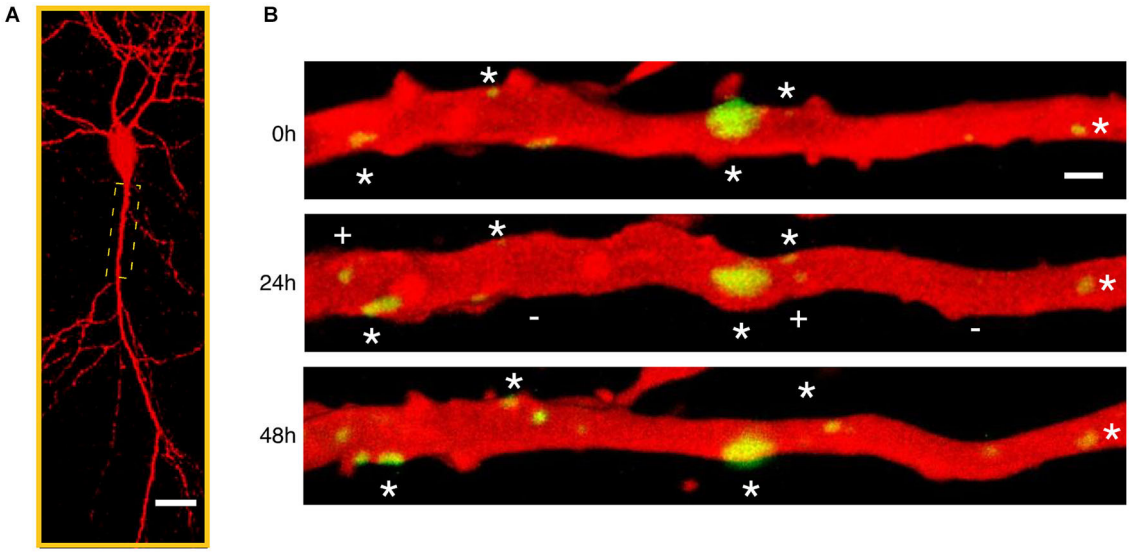

FIGURE 3 | Time-lapse imaging of gephyrin containing inhibitory synapses. (A) Low magnification confocal microscope view of a CA1 pyramidal neuron in a mature hippocampal organotypic culture imaged after biolistic transfection with Red Fluorescent Protein to reveal neuronal structure and Green Fluorescent Protein tagged gephyrin to visualize the postsynaptic component of inhibitory synapses. **(B)** Repetitive laser scanning confocal imaging of an apical proximal dendrite (boxed region in **A**) taken every 24 h during 3 consecutive days. A large fraction of gephyrin-containing inhibitory synapses (green) are stable throughout the imaging period (stars) despite the high level of structural variability. In addition, gephyrin clusters appear (plus (+) sign) and disappear (minus (–) sign) at different dendritic locations suggesting continuous formation and elimination of inhibitory synapses.

experience-dependent inhibitory synapse formation critically depends on NPAS4-mediated gene expression and results in differential modulation of somatic and dendritic inhibition (Bloodgood et al., 2013). Interestingly, NPAS4 is a critical mediator of activity dependent expression of the neurotrophin Brain Derived Neurotrophic Factor (BDNF) that regulates developmental and activity dependent formation and elimination of inhibitory synapses (Marty et al., 2000; Berghuis et al., 2004; Jovanovic et al., 2004; Kohara et al., 2007). Structural plasticity of GABAergic synapses is at least in part controled by a postsynaptic mechanism that links neuronal activity with alteration in nuclear gene expression.

In a pioneering attempt to elucidate the molecular mechanisms of inhibitory synaptic plasticity, Nusser and colleagues (Nusser et al., 1998) used a combination of quantal analysis of evoked inhibitory postsynaptic currents with quantitative immunogold labeling of synaptic $GABA_A$ Rs in hippocampal granule cells. They found that an increase in amplitude of synaptic currents corresponded to a proportional increase in the number of $GABA_A$Rs at inhibitory synapses on somata and axon initial segments of hippocampal granule cells. The increased density of $GABA_A$ Rs was accompanied by an enlargement of synaptic area and presynaptic boutons (Nusser et al., 1998). Although our knowledge of the mechanisms of activity-dependent structural plasticity of inhibitory synapses is still limited, this study suggests that, like excitatory synapses, inhibitory synaptic plasticity might not only depend on changes in the biophysical properties of the channels, but might also involve a structural reorganization of its inhibitory postsynaptic density, by altering the number of channels and the necessary structural components. Recent research shows that gephyrin forms dynamic domains within the inhibitory PSD that change size, form and localization in minutes (Specht et al., 2013). In the the hexagonal bidimensional lattice formed by gephyrin at the postsynaptic membrane, every single gephyrin interacts with GABA receptors in a one to one ratio approximately (Specht et al., 2013). Increasing synaptic gephyrin would then create new slots for allocating inhibitory receptors. Interestingly, functional and structural plasticity of excitatory synapses share a complete set of molecular machinery that allows coordinated changes in synaptic strength and architecture (Lüscher et al., 2000). Increasing our knowledge of the molecular mechanisms that regulate clustering of gephyrin and trafficking of $GABA_A$ R at inhibitory synapses will surely increase our understanding of molecular mechanisms that regulate structural plasticity of GABAergic synapses.

ACTIVITY DEPENDENT REGULATION OF GEPHYRIN FUNCTION

One of the most intensively studied molecule involved in the inhibitory synapse function is gephyrin, the major scaffold protein of the inhibitory synapse. Crystallographic techniques show that gephyrin oligomerizes via G-domain trimerization and E-domain dimerization forming an hexagonal submembrane lattice (Xiang et al., 2001). Gephyrin undergoes several post-translational modifications that affect its clustering and the interactions with $GABA_A$Rs and a variety of regulatory proteins, signaling complexes and cytoskeleton (Tyagarajan and Fritschy, 2014). Dejanovic et al. (2014) have recently identified gephyrin palmitoylation as a mechanism of gephyrin synaptic targeting. As for PSD95 in excitatory synapses, gephyrin palmitoylation is essential for the interaction with cell membrane and therefore

facilitates its postsynaptic clustering at GABAergic synapses. Gephyrin palmitoylation is dependent of synaptic activity since the application of bicuculline that blocks $GABA_A$ Rs and increases network activity reduces gephyrin palmitoylation. In contrast, GABA application increases gephyrin palmitoylation (Dejanovic et al., 2014). Additionally, synaptic activity may alter gephyrin clustering via modulation of nitric oxide (NO) synthesis by neuronal NO synthase (Dejanovic and Schwarz, 2014).

Phosphorylation is a critical regulator of gephyrin function (Moss et al., 1995; Zacchi et al., 2014). Some of the signaling cascades that modulate gephyrin phosphorylation and GABAergic structural plasticity could represent a cross-road between excitatory and inhibitory synapse function, thus providing a mechanism of extreme importance to maintain the homeostasis of synaptic networks. Clear examples for such common molecular players are Glycogen Synthase Kinase 3 (GSK3β) and extracellular signal-regulated protein kinase/mitogen-activated protein kinase (ERK). At excitatory synapses, GSK3β phosphorylates PSD95 and modulates excitatory synaptic plasticity in an N-Methyl-D-Aspartate (NMDA) receptor-dependent manner by inducing α-Amino-3-hydroxy- 5-Methyl-4-isoxazolepropionic acid (AMPA) receptor internalization and thus LTD (Nelson et al., 2013) while ERKs boost excitatory synaptic and structural plasticity at different levels (Zhu et al., 2002; Patterson et al., 2010). At inhibitory synapses, gephyrin phosphorylation by both GSK3β and ERK1 at at Ser 270 and Ser 268 respectively modulates the structure and function of GABAergic synapses by altering the number and size of gephyrin clusters (Tyagarajan et al., 2011b, 2013). Glycogen Synthase Kinase 3-dependent phosphorylation decreases gephyrin cluster size, whereas phosphorylation by ERK decreases size and density of postsynaptic gephyrin clusters and is critical for the Ca2+-dependent cysteine protease (calpain-1) degradation of gephyrin. Phosphorylation of gephyrin by GSK3β and ERK1 is accompanied by parallel decrease in GABAergic mIPSCs. ERK and GSK3β activity are tightly regulated by neuronal activity making gephyrin phosphorylation a key mechanism for the coordination of structural remodeling of GABAergic synapses and network activity levels.

CA2+/CALMODULIN DEPENDENT PROTEIN KINASE II REGULATES GABAERGIC SYNAPSE FUNCTION AND STRUCTURE

Trafficking and stability of $GABA_A$ Rs can be modulated by direct phosphorylation of the channel subunits (Vithlani et al., 2011). Several kinases target $GABA_A$ R such as Protein Kinase A (Brandon et al., 2003; Jovanovic et al., 2004), Protein Kinase C (Brandon et al., 2002) and Ca2+/calmodulin-dependent protein kinase II (CaMKII; McDonald and Moss, 1997). CaMKII directly phosphorylates α1, β2, β3, and γ2 subunits (McDonald and Moss, 1997; Churn et al., 2002; Houston et al., 2009; Petrini et al., 2014). Activation of the NMDA subtype of glutamate receptors has been linked to potentiation of $GABA_A$ R-mediated currents in different brain regions through activation of CaMKII (Marsden et al., 2007; Petrini et al., 2014). In addition, CaMKII translocates from dendritic spines to inhibitory synapses upon weak chemical stimulation by NMDA and promotes the insertion of $GABA_A$ Rs to inhibitory synapses and enhancement of inhibitory transmission (Marsden et al., 2010). Are these functional changes accompanied by structural remodeling? A recent published work by Petrini et al. (2014) found that the chemical inhibitory long term potentiation (iLTP) triggered by NMDA application (up to 30 min) requires CaMKII-dependent phosphorylation of $GABA_A$ R subunit β3 at serine 383 that in turn promotes the synaptic recruitment of gephyrin from extrasynaptic sites. The increase of gephyrin at synapses is not explained by de novo synthesis, but rather by a regulation of the mechanisms that control the redistribution of gephyrin. In turn, using single-particle tracking of quantum dots labeled $GABA_A$Rs, they found that gephyrin recruitment at the synapses stabilizes $GABA_A$ Rs. The recruitment of gephyrin by an activity dependent phosphorylation of $GABA_A$ R subunit represents a way in which functional changes are coordinated with structural changes.

CELL ADHESION MOLECULES AND ACTIVITY DEPENDENT REGULATION OF GABAERGIC SYNAPSE

Gephyrin clustering at GABAergic synapses requires its interaction with a neuron specific Guanine Nucleotide Exchange Factor (GEF) Collybistin (CB; Kins et al., 2000; Harvey et al., 2004; Papadopoulos et al., 2007). CB is expressed specifically in neurons and activates cell division control protein 42 homologue (Cdc42; Xiang et al., 2006) and the small Rho-like GTPase TC10 (Mayer et al., 2013) that in turn regulate cluster formation and the aggregation $GABA_A$ Rs (Poulopoulos et al., 2009; Tyagarajan et al., 2011a). Inhibitory synapse formation, maturation, maintenance and function are also regulated by synaptic cell adhesion molecules (CAMs; Yamagata et al., 2003; Gerrow and El-Husseini, 2006). At inhibitory synapses, one of the most prominent CAMs belongs to the Neuroligin family (Craig and Kang, 2007; Südhof, 2008). Neuroligin 2 (NL2) is specifically localized at inhibitory synapses (Varoqueaux et al., 2004) where it modulates their formation, maturation and function (Graf et al., 2004; Chih et al., 2005; Chubykin et al., 2007). NL2 deficient mice lack postsynaptic specialization at perisomatic inhibitory synapses (Poulopoulos et al., 2009) and show decreased inhibitory synaptic transmission (Chubykin et al., 2007). Through a conserved cytoplasmatic domain, NL2 binds gephyrin (Poulopoulos et al., 2009) while an extracelullar motif mediates NL2 trans-synaptic interaction with presynaptic Neurexins (NRXs) boosting inhibitory presynaptic axonal differentiation during development (Chih et al., 2005). The interaction with the scaffold protein gephyrin brings NL2 close to GABA receptors and other gephyrin binding proteins (Fritschy et al., 2012). NRXs are CAMs that have two isoforms, α-NRXs and β-NRXs (Südhof, 2008). α-NRXs are expressed primarily at GABAergic synapses, whereas β-NRXs are localized at both excitatory and inhibitory synapses (Chih et al., 2005) where they form a dense transynaptic assembly (Tanaka et al., 2012). Interestingly, β-NRX1 has high turn over rate in presynaptic membranes and is stabilized by neuronal activity and GABA release (Fu and Huang, 2010). In addition, NRXs directly interact with $GABA_A$ Rs, thus modulating GABAergic transmission in a NL independent fashion (Zhang et al., 2010).

GABA dependent stabilization of presynaptic NRXs may represent a mechanism for activity dependent GABAergic synapse remodeling. NRXs stabilized by local GABA release (Fu and Huang, 2010) drive the clustering of NL2 postsynaptically.

Neuroligin 2 interaction with gephyrin brings CB close to NL2, where CB/NL2 interaction releases SH3 domain and activates CB (Poulopoulos et al., 2009). Interaction with $GABA_AR$ α2 subunit may also activate CB (Tretter et al., 2008). Activated CB leads gephyrin and $GABA_A$ Rs to the postsynaptic membrane and further stabilizes them at the synapses. Neuroligin 2 initiates clustering of other molecules essential for synapse function (Lévi et al., 2002; Woo et al., 2013; Pribiag et al., 2014) strengthening in this way synaptic adhesion. Finally, NL2 intracellular interactions with γ2 subunit of $GABA_AR$ stabilize both molecules at inhibitory synapses (Dong et al., 2007). Thus, GABA unchained downstream signaling may play a role in coordinating the formation and remodeling of the pre- and postsynapses (Fritschy et al., 2008; Tretter et al., 2012).

FUNCTIONAL ROLE AND SPECIFICITY

The study of structural plasticity of glutamatergic synapses has focused on the relationship between morphological parameters of dendritic spines and physiological properties of its excitatory synapse (Nimchinsky et al., 2002; Bourne and Harris, 2008; Kasai et al., 2010). Recent studies have shown that although inhibitory post-synapses do not have such morphological fingerprint, fluorescently tagged gephyrin can be used to visualize inhibitory synapses and track dynamic changes (Dobie and Craig, 2011; Chen et al., 2012; van Versendaal et al., 2012). Indeed, virtually all gephyrin clusters detected at the optical level using fluorescent microscopy had a correlate with a GABAergic synapse detected at the ultrastructural level using EM (Chen et al., 2012; van Versendaal et al., 2012). Although the functional correlation between optically measurable parameters such as size and intensity of gephyrin clusters and inhibitory synapse function is currently unknown, EM studies have shown that increased synaptic strength in inhibitory synapses produces a coordinated insertion of $GABA_ARs$ and enlargement of the PSD (Nusser et al., 1998). Whether such structural rearrangement can be visualized at the optical level using fluorescently-tagged gephyrin or other inhibitory synapse fluorescent markers requires further research that will provide invaluable information about structure-function relationship in inhibitory synapses.

During brain development, when structural dynamism of GABAergic synapses is high, the functional consequences of inhibitory synaptic rearrangements are determined by the depolarizing effects that activation of $GABA_ARs$ has on postsynaptic target cells (Cherubini et al., 1991). In adulthood, however, coordination of the amount of excitatory and inhibitory inputs becomes an essential function of structural plasticity of GABAergic synapses. Both perisomatic (Nusser et al., 1998; Lushnikova et al., 2011) and dendritic inhibitory synapses (Knott et al., 2006; Jasinska et al., 2010; Chen et al., 2012; van Versendaal et al., 2012) show high levels of structural plasticity, suggesting that excitation/inhibition balance could be controled independently in different subcellular compartments. Indeed, recent reports show that active excitatory synaptic inputs are not randomly distributed along dendritic arbors in principal cells neurons (Kleindienst et al., 2011; Makino and Malinow, 2011; Takahashi et al., 2012). The ability of inhibitory synapses to target specific subcellular compartments could allow local control of such clustered inputs. In addition, an homeostatic role is a reasonable interpretation for studies showing simultaneous rearrangement of perisomatic inhibitory and excitatory synapses in response to patterns of activity known to induce potentiation of glutamatergic synaptic transmission (Nusser et al., 1998; Bourne and Harris, 2011; Lushnikova et al., 2011). In the barrel cortex, increases and decreases in whisker-induced neuronal activity are directly related to the formation or elimination of GABAergic spine synapses respectively (Micheva and Beaulieu, 1995; Knott et al., 2002). This form of GABAergic structural plasticity may participate in the homeostatic adjustment of circuit activity levels after long-term changes in somatosensory evoked synaptic activity. It is increasingly clear that patterns of synaptic activity that induce structural rearrangements of inhibitory circuits also induce plasticity (functional and/or structural) of excitatory synapses. Functional plasticity of GABAergic synapses has been shown to modulate excitatory neurotransmission and neuronal output (Saraga et al., 2008; Wang and Maffei, 2014). This could reflect network's requirement for a coordinated plasticity of inhibitory and excitatory inputs to maintain homeostasis, keep excitation/inhibition balance and prevent abnormal levels of activity.

One of the main components of LTP in the hippocampus is the increase in the efficacy of coupling between excitatory postsynaptic depolarization and spiking activity (Linden, 1999; Lu et al., 2000). Since perisomatic inhibition plays an essential role in determining the time window for spike generation (Pouille and Scanziani, 2001), increased inhibition through structural rearrangements of perisomatic synapses after plasticity inducing network activity may help in ensuring precise temporal synaptic integration. Addition or removal of GABAergic spine synapses in close proximity glutamatergic synapses in dendritic spines of cortical neurons allow the control of excitatory inputs through shunting inhibition (Maccaferri, 2005) and modulation of calcium signaling by near coincident activation of the GABAergic and excitatory inputs impinging on a particular spine (Chiu et al., 2013; Hayama et al., 2013) and it has the potential to affect processes of local, branch or dendritic segment specific computation (Pérez-Garci et al., 2006). In addition, dendrite inhibition can be very effective in damping neuronal activity by long-range shunting of excitatory inputs close to the soma. This means that plasticity of a few strategically located synapses could have important consequences for determining neuronal output (Gidon and Segev, 2012). In addition to its homeostatic role, inhibitory synapse structural plasticity may refine the synaptic basis of computational operations performed by hippocampal and cortical networks.

Experience shapes the formation and function of neuronal circuits during critical periods in early life (Berardi et al., 2000; Hensch, 2004). It seems increasingly clear that during these sensitive periods, special plasticity mechanisms that have a much smaller prevalence during adulthood are potentiated. In the visual cortex, where critical period has been extensively studied, the transition in and out of this critical period has been demonstrated to be under a strict control of the GABAergic system with a prominent role of perisomatic inhibition (Hensch, 2005). Changes in the rules of functional GABAergic synapse plasticity in cortical synapses that take place after the developmental switch

in GABA synaptic signal polarity may play a prominent role in the transition into these periods of enhanced plasticity (Lefort et al., 2013). In addition, developmental and activity driven structural plasticity of GABAergic synapses may have important consequences for critical periods. In line with this, several studies have demonstrated that cortical sensory areas undergo an early disinhibition upon sensory deprivation (Micheva and Beaulieu, 1995; Keck et al., 2011; Chen et al., 2012; van Versendaal et al., 2012). It is likely that GABAergic synapse elimination is fundamental in allowing subsequent plastic changes in the cortex that may affect glutamatergic transmission. It is tempting to speculate that the interaction between GABAergic and glutamatergic synapses could involve a bidirectional crosstalk that include signals from the excitatory synapse that regulate GABAergic synapse strength and persistence (Marsden et al., 2010). Perineuronal nets (PNNs) are well organized structures formed by extracellular matrix molecules condensed around cell body and proximal dendrites of some types of neurons (Kwok et al., 2011). PNNs are formed by aggregation of heavily glycosilated proteins (proteoglycans) and, in the cortex, are preferentially associated with GABAergic cells (Morris and Henderson, 2000). Interestingly, PNNs are emerging as key structural regulators of INs plasticity (Wang and Fawcett, 2012). PNNs expression restricts neuronal plasticity by stabilizing synaptic connections and inhibiting activity dependent changes in neurotransmission (Berardi et al., 2004). Interestingly, disruption of PNNs con reactivate plasticity in some brain areas (Gogolla et al., 2009) and allows the reactivation of critical periods in the adult brain (Pizzorusso et al., 2002).

Changes in GABAergic transmission are essential for certain forms of memory and learning (Cui et al., 2008) but the information about the involvement of GABAergic synapse structural plasticity in network mechanisms of memory is still scarce. Some experiments have shown that learning-related behavior can drive long-lasting inhibitory synapse formation and elimination (Jasinska et al., 2010; Bloodgood et al., 2013) that may be responsible for durable changes in network connectivity underlying learning and memory. Behaviorally induced structural plasticity does not involve the general population of inhibitory synapses but differentially affects synapses impinging onto dendrites, spines or perisomatic compartment (Bloodgood et al., 2013). As a consequence of this differential regulation, experience may change the spread of inhibitory input among the different compartments and may affect information processing (Miles et al., 1996). Interestingly, selective deletion of Npas4 gene that codes for an activity regulated transcription factor responsible for differential remodeling of hippocampal dendritic and somatic GABA synapses in response to spatial exploration (Bloodgood et al., 2013), blocks hippocampal-dependent contextual learning (Ramamoorthi et al., 2011). Although Npas4 has been shown to control numerous genetic pathways that regulate both excitatory and inhibitory synapse function (Spiegel et al., 2014), its role in contextual memory formation could be at least in part mediated by an activity dependent remodeling of GABAergic synapses.

CONCLUSIONS AND FUTURE DIRECTIONS

In this review we have emphasized the role of synaptic activity in the remodeling of GABAergic synapse structure and discussed the possible roles of structural plasticity in sensory processing and memory formation. Although there is substantial experimental evidence of activity driven structural plasticity of GABAergic synapses, we have only partial knowledge of the implications of this type of plasticity for the function of inhibitory synapses and circuits and the molecular mechanisms that regulate different aspects of GABAergic synapse remodeling. In particular, several questions remain open for future investigation: (i) what are the structural determinants of GABAergic synapse function? (ii) what is the driving force for remodeling of GABA synapses, GABAergic or glutamatergic neurotransmission (or both)? (iii) how is the persistence of GABAergic synapses controled? (iv) how is structural plasticity of GABAergic and Glutamatergic synapses coordinated? (v) why spine inhibitory synapses are more dynamic than shaft inhibitory synapses? Addressing all these questions will surely advance our knowledge of the brain mechanisms of plasticity and define the precise roles of inhibitory synapse remodeling in the neuronal adaptation to experience, and in particular, for learning and memory.

ACKNOWLEDGMENTS

The authors would like to thank Dominique Muller for intellectual and financial support and Irina Nikonenko for providing the EM image and critically reading the manuscript. Work in Dominique Muller's lab is financed by SNF grant Sinergia and grant 310030B-144080.

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

Received: 21 August 2014; accepted: 28 September 2014; published online: 27 October 2014.

Citation: Flores CE and Méndez P (2014) Shaping inhibition: activity dependent structural plasticity of GABAergic synapses. Front. Cell. Neurosci. ***8****:327. doi: 10.3389/fncel.2014.00327*

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

frontiers in
CELLULAR NEUROSCIENCE

REVIEW ARTICLE
published: 23 September 2014
doi: 10.3389/fncel.2014.00300

Diffusion dynamics of synaptic molecules during inhibitory postsynaptic plasticity

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The plasticity of inhibitory transmission is expected to play a key role in the modulation of neuronal excitability and network function. Over the last two decades, the investigation of the determinants of inhibitory synaptic plasticity has allowed distinguishing presynaptic and postsynaptic mechanisms. While there has been a remarkable progress in the characterization of presynaptically-expressed plasticity of inhibition, the postsynaptic mechanisms of inhibitory long-term synaptic plasticity only begin to be unraveled. At postsynaptic level, the expression of inhibitory synaptic plasticity involves the rearrangement of the postsynaptic molecular components of the GABAergic synapse, including $GABA_A$ receptors, scaffold proteins and structural molecules. This implies a dynamic modulation of receptor intracellular trafficking and receptor surface lateral diffusion, along with regulation of the availability and distribution of scaffold proteins. This Review will focus on the mechanisms of the multifaceted molecular reorganization of the inhibitory synapse during postsynaptic plasticity, with special emphasis on the key role of protein dynamics to ensure prompt and reliable activity-dependent adjustments of synaptic strength.

Keywords: $GABA_A$ receptors, GABAergic plasticity, scaffold proteins, single particle tracking, gephyrin, lateral diffusion, intracellular trafficking, phosphorylation

INTRODUCTION

γ-aminobutyric acid (GABA) receptors mediate the majority of inhibitory signals in the brain. GABAergic inhibition consists of a fast and precisely timed component generated by the vesicular release of GABA in the synaptic cleft (phasic inhibition), and of a persistent tonic conductance due to receptor activation by ambient GABA (tonic inhibition) (Farrant and Nusser, 2005; Olsen and Sieghart, 2008). While phasic inhibitory transmission is mainly mediated by the activation of α1-3β2-3γ $GABA_A$ receptors ($GABA_A$Rs) clustered at synapses, tonic conductance arises from extrasynaptic $GABA_A$Rs typically composed of α1/4/6βδ and α5βγ. It is well known that inhibitory transmission is crucial to tune neuronal excitability and to regulate network integration. In particular, at network level, inhibitory synaptic signals are fundamental for generating coherent oscillations and selection of cell assemblies (Bartos et al., 2007; Klausberger and Somogyi, 2008; Royer et al., 2012). As such, GABAergic inhibition controls higher cognitive functions in the brain and lies at the basis of some neurological disorders when impaired (Lewis et al., 2012; Katona et al., 2014). Over the last decades, accumulating evidence has revealed that both phasic and tonic inhibitory signals can be plastic, thus raising additional possibilities for the modulation of network activity and neuronal circuit refinement (Kano et al., 1992; Saliba et al., 2012; Bright and Smart, 2013). The emerging role of inhibitory synaptic plasticity in higher brain functions has provided a strong drive towards the investigation of the underlying cellular, structural and molecular determinants (Frotscher et al., 1990; Kozhedub and Knipst, 1997). As a consequence, a variety of "inhibitory plasticities" can be identified in different brain regions such as cerebellum, hippocampus, visual cortex, ventral tegmental area, lateral amygdala (Kano et al., 1992; Marsicano et al., 2002; Patenaude et al., 2003; Maffei et al., 2006; Nugent et al., 2007; Heifets et al., 2008). However, a comprehensive knowledge of the mechanisms that lead to activity-dependent changes of inhibitory synaptic strength has been limited by the strong diversity of (i) inhibitory interneuron cell types; (ii) inhibitory synapses along the dendritic arbor and the soma; and (iii) $GABA_A$ receptor subtypes. Some of the best characterized forms of inhibitory synaptic plasticity depend on changes in presynaptic GABA release (McBain and Kauer, 2009; Castillo et al., 2011). Messengers such as endocannabinoids, BDNF or NO, released from the postsynaptic cell in activity-dependent manner, retrogradely diffuse to the presynaptic terminal, where they modulate the amount of GABA released in the cleft, thereby inducing the depression or potentiation of inhibitory synaptic strength (Nugent et al., 2007; Heifets et al., 2008; Sivakumaran et al., 2009). Other forms of inhibitory synaptic plasticity are expressed postsynaptically with persistent modifications of the abundance (Nusser et al., 1998; Kilman et al., 2002; Marsden et al., 2007; Kurotani et al., 2008; Bannai et al., 2009; Muir et al., 2010; Niwa et al., 2012; Saliba et al., 2012; Nahmani and Turrigiano, 2014; Petrini et al., 2014), assortment (Houston et al., 2008; Rajalu et al., 2009) and gating (Moss et al., 1995; Jones and

Westbrook, 1997; Houston et al., 2008) of postsynaptic $GABA_A$ receptors. Such changes depend on a coordinated sequence of dynamic events that tune receptor delivery to, stabilization at, and removal from synapses. Additionally, at postsynaptic level, activity-dependent changes of the chloride transporters can affect inhibitory synaptic currents by altering the postsynaptic chloride driving force (Rivera et al., 1999; Sun and Murali, 1999). Moreover, intracellular chloride has been reported to act as a biochemical messenger by influencing the expression of different $GABA_A$R subtypes (Succol et al., 2012). Overall, postsynaptic forms of inhibitory synaptic plasticity rely on complex processes leading to the active reorganization of GABAergic synapses at molecular level. Of note, some of the determinants of postsynaptic inhibitory plasticity are shared with excitatory synapses, suggesting that evolutionarily conserved mechanisms adjust synaptic strength through the coordinated control of postsynaptic receptor availability.

This Review will focus on the key role of molecule dynamics in the expression of inhibitory postsynaptic plasticity, with special emphasis on the activity-regulated changes in receptor trafficking, receptor lateral mobility and scaffold protein dynamic organization. Moreover, some aspects of the postsynaptic plasticity of excitatory and inhibitory synapses will be compared in order to highlight convergent points in the regulation of cell excitability.

ROLE OF $GABA_A$ RECEPTOR INTRACELLULAR TRAFFICKING IN THE EXPRESSION OF INHIBITORY SYNAPTIC PLASTICITY

The initial demonstrations that $GABA_A$Rs are brought to the neuronal surface by exocytosis and removed by clathrin-mediated endocytosis (Tehrani and Barnes, 1993; Kittler et al., 2000) represent the first indications that neurotransmitter receptors are not fixed at the neuronal membrane but exchange between surface and intracellular compartments. Since then, many laboratories have contributed to elucidate the multiple steps of $GABA_A$Rs intracellular trafficking and have extended their studies to other neurotransmitter receptors (Maloteaux and Hermans, 1994; Luscher et al., 1999; Kittler et al., 2000; Park et al., 2004; Bogdanov et al., 2006). The large number of proteins that assist $GABA_A$R intracellular dynamics (namely their exocytic/endocytic pathways and their sorting to degradation or recycling) will not be addressed here, since they have been extensively discussed in excellent reviews (Chen and Olsen, 2007; Arancibia-Cárcamo and Kittler, 2009; Jacob et al., 2009; Luscher et al., 2011; Vithlani et al., 2011).

Receptor intracellular trafficking ensures receptor renewal in basal conditions (Charych et al., 2004; Kittler et al., 2004; Vithlani et al., 2011; Huganir and Nicoll, 2013); however, it also underlies many forms of synaptic plasticity at inhibitory and excitatory synapses by dynamically regulating surface receptor availability (Luscher et al., 2011; Huganir and Nicoll, 2013). At GABAergic synapses, postsynaptically-expressed potentiation of long-term potentiation inhibition (iLTP) depends on GABARAP-mediated increase of $GABA_A$R exocytosis that in turn promotes receptors accumulation at the postsynaptic density (PSD), as observed in cultured neurons, in slices and *in vivo* (Nusser et al., 1998; Marsden et al., 2007; Kurotani et al., 2008; Nahmani and Turrigiano, 2014; Petrini et al., 2014). Conversely, inhibitory long-term depression (iLTD) has been correlated with reduced availability of $GABA_A$Rs at synapses, although no consensus has been achieved yet whether this is due to altered $GABA_A$R internalization or to receptor dispersal from the synapse (Kurotani et al., 2008; Bannai et al., 2009; Muir et al., 2010). Indeed, the cell-specific blockade of $GABA_A$R endocytosis prevents the depression of inhibition in slices of the primary visual cortex (Kurotani et al., 2008), while it does not affect the expression of iLTD in hippocampal neuronal cultures (Bannai et al., 2009; Muir et al., 2010). Similarly, at excitatory synapses, a large body of evidence describes increased AMPA receptor exocytosis as one of the main mechanisms underlying several forms of long-term potentiation (LTP) and reduced availability of AMPA receptors to be causal for long-term depression (LTD) (reviewed in Huganir and Nicoll, 2013).

Another key element of receptor intracellular trafficking that contributes to the regulation of surface receptor number and that can play a role for the expression of postsynaptic plasticity is receptor endocytic sorting (Luscher et al., 2011; Vithlani et al., 2011). In fact, by determining the fate of endocytosed receptors towards receptor lysosomal degradation or recycling to the surface, receptor endocytic sorting can set the number of receptors actively involved in receptor turnover. Hence, the preferential routing of the receptors to the recycling or lysosomal pathway would sustain increased or reduced surface receptor number during synaptic potentiation or depression, respectively (Kittler et al., 2004; Arancibia-Cárcamo et al., 2009; Mabb and Ehlers, 2010). It should be noted that the delivery of recycling receptors to the surface can be faster than that of newly synthesized receptors, which has been estimated in a time range spanning from few minutes to hours, thus providing a faster regulation of surface receptor abundance (Connolly et al., 1999; Bogdanov et al., 2006; Renner et al., 2008).

The key role of receptor trafficking in the expression of synaptic plasticity, initially demonstrated *in vitro*, has been confirmed *in vivo* by experiments addressing ecitatory and inhibitory synaptic plasticity in the barrel cortex during cortical map formation and sensory experience (Lu et al., 2003; Clem and Barth, 2006), during fear-conditioning in the amygdala and in the nucleus accumbens (Chhatwal et al., 2005; Schierberl et al., 2011), in the medial prefrontal cortex induced by cocaine (Bellone and Luscher, 2006; Ghasemzadeh et al., 2011), in the visual cortex (Frenkel et al., 2006; Nahmani and Turrigiano, 2014), and in the hippocampus (Lee et al., 2003; Tretter et al., 2009).

$GABA_A$ RECEPTOR POST-TRANSLATIONAL MODIFICATIONS AND SYNAPTIC PLASTICITY

There is compelling evidence that the phosphorylation and dephosphorylation of $GABA_A$Rs are key events for the expression of inhibitory synaptic plasticity (Comenencia-Ortiz et al., 2014). In fact, the surface expression of $GABA_A$Rs depends on the activity of several kinases and phosphatases (such as CaMKII, PKA, PKC, Src, Akt, calcineurin) that tightly modulate receptor intracellular trafficking by acting on specific sites of receptor intracellular domains. It has been initially demonstrated that

the intracellular application of preactivated CaMKII potentiates GABAergic currents evoked with exogenous GABA pulses by increasing the phosphorylation of Ser 383 of $GABA_A$ receptor β3 subunit (Houston et al., 2007). Recently, the CaMKII-mediated phosphorylation of Ser 383 on β3 subunit has been demonstrated to be an essential requirement for the LTP of inhibitory synaptic currents as it promotes the exocytosis and the postsynaptic accumulation/immobilization of $GABA_AR$ at synapses (Petrini et al., 2014). Along the same lines, the serine/threonine kinase Akt increases the number of surface α1 subunit-containing $GABA_ARs$ by phosphorylating Ser 410 of the β2 subunit, thereby enhancing inhibitory synaptic transmission in the hippocampus *in vitro* and *in vivo* (Wang et al., 2003b). Akt-mediated larger delivery of $GABA_ARs$ to the neuronal surface has also been observed in midbrain ventral tegmental area neurons following stress stimuli that activate δ opioid receptors and elicit postsynaptic potentiation of $GABA_A$-mediated inhibitory currents (Margolis et al., 2011).

$GABA_AR$ phosphorylation can also tune inhibitory synaptic strength by regulating receptor endocytosis through the modulation of $GABA_ARs$ interactions with the endocytic machinery. Indeed, the binding motifs for the endocytic adaptor protein AP2 on $GABA_AR$ β and γ2 subunits incorporate sites for phosphorylation by PKA, PKC, Akt and Src and dephosphorylation by protein phosphatase 1 (PP1), PP2A and calcineurin (Brandon et al., 2002; Wang et al., 2003a; Jovanovic et al., 2004; Kittler et al., 2008). Therefore, the phosphorylation of the these residues (or of adjacent ones) precludes the binding of AP2 to the receptors, resulting in a negative regulation of $GABA_AR$ endocytosis (Kittler et al., 2000, 2008; Herring et al., 2003), with the consequent increase of surface $GABA_AR$ number (Kittler et al., 2008; Jacob et al., 2009; Smith et al., 2012). Conversely, the dephosphorylation of the residues involved in $GABA_AR$-AP2 interaction favors $GABA_AR$ internalization and decreases the abundance of $GABA_ARs$ in the neuronal membrane. For instance, in the medial prefrontal cortex, the reduced GABAergic inhibition observed after cocaine withdrawal is the result of increased PP2A activity that promotes the dephosphorylation of Ser 408/409 on $GABA_AR$ β3 subunit and enhances receptor internalization (Jovanovic et al., 2004; Lu et al., 2010). In the CA1 region of the hippocampus, it has been proposed that tetanus-induced LTD of unitary IPSCs might rely on a reduction of functional $GABA_AR$ number at synapses following the dephosphorylation of γ2 subunit by calcineurin (Wang et al., 2003a). Recently, GABAergic plasticity observed after *in vivo* Ethanol (EtOH) administration (Liang et al., 2007; Olsen and Spigelman, 2012) has been explained by promoted α4βδ $GABA_AR$ endocytosis due to the interaction of $GABA_AR$ δ subunit with the AP2 machinery (Gonzalez et al., 2012).

Remarkably, the phosphorylation of $GABA_ARs$ has been also involved in the plasticity of tonic inhibition, similarly to the plasticity of synaptic inhibition. In fact, also the abundance of surface extrasynaptic receptors has been related to phosphorylation-dependent modulation of $GABA_AR$ trafficking. For instance, the CaMKII-dependent phosphorylation of Ser 383 on β3 subunit, elicited by the activation of L-type voltage-gated Ca^{2+} channels, favors the exocytosis of α5-containing $GABA_ARs$, thus enhancing the non-synaptic component of GABAergic inhibition in cultured hippocampal neurons (Saliba et al., 2012). Analogously, the activation of PKC induces the phosphorylation of Ser 443 on α4 subunit and of Ser 408/409 on β3 subunit of $GABA_AR$, leading to the promoted surface delivery and increased membrane stability of α4-containing receptors (Abramian et al., 2010). More recently, this mechanism has been implicated in the increase of extrasynaptic α4-containing $GABA_ARs$ underlying the potentiation of tonic inhibition induced by neurosteroids (Abramian et al., 2014). Overall, the experimental evidence described above define a common rule for the plasticity of phasic and tonic inhibition, namely that the phosphorylation stabilizes and dephosphorylation destabilizes $GABA_ARs$ at the neuronal surface. However, the phosphorylation of $GABA_AR$ has also been lately demonstrated to promote $GABA_ARs$ endocytosis, thus oppositely contributing to the plasticity of tonic inhibition. Namely, the phosphorylation of Ser 410 on β2 subunit by PKC reduces the surface expression of extrasynaptic α4β2δ $GABA_ARs$, resulting in the depression of tonic inhibition in the dentate gyrus region of the hippocampus and in the thalamus (Bright and Smart, 2013). It is possible that the assortment of the α4 subunit with the β2 or β3 subunit might differentially regulate the phosphorylation and trafficking of α4-containing $GABA_ARs$, resulting in the PKC-mediated depression or potentiation of tonic inhibition, respectively (Bright and Smart, 2013; Abramian et al., 2014). Another evidence that specific phosphorylation of $GABA_AR$ subunits differentially affect the strength of inhibition depending on the $GABA_AR$ subunit assortment is provided by the phosphorylation of the Ser 410 on β2 subunit. If the phosphorylated β2 subunit is within a α1subunit-containing receptor, the membrane stability of $GABA_ARs$ in increased, leading to the potentiation of GABAergic synaptic transmission; on the contrary, if the phosphorylated β2 subunit is assembled with the α4 subunit it mediates the depression of inhibitory tonic currents by reducing the number of surface receptor (Wang et al., 2003b; Bright and Smart, 2013). Of note, the phosphorylation of $GABA_ARs$ can also tune inhibitory synaptic strength by directly affecting the microscopic gating of the $GABA_ARs$, hence providing a potential additional level of receptor modulation during synaptic plasticity (Moss et al., 1995; Jones and Westbrook, 1997; Brandon et al., 2000). Interestingly, Tretter et al. (2009) have studied the behavioral impact of inhibitory synaptic plasticity induced by phospho-regulated changes of $GABA_AR$ intracellular trafficking. Knock-in mice in which the principal sites of tyrosine phosphorylation within $GABA_AR$ γ2 subunit (Y365, Y367) have been mutated to phenylalanine have been probed for some cognitive aspects. The Y365/7F mice exhibit aberrant endocytic pathway due to the compromised $GABA_AR$-AP2 interaction, leading to increased accumulation of synaptic $GABA_AR$ on pyramidal neurons of the CA3 region of the hippocampus. The resulting potentiated inhibitory transmission in these Y365/7F mice correlates with specific CA3 hippocampal-dependent deficits in spatial object recognition, such as the inability to discriminate between displaced and non-displaced objects, despite intact object recognition memory (Tretter et al., 2009).

Besides receptor phosphorylation, other post-translational modifications such as ubiquitination and palmitoylation have been implicated in the control of the surface expression of

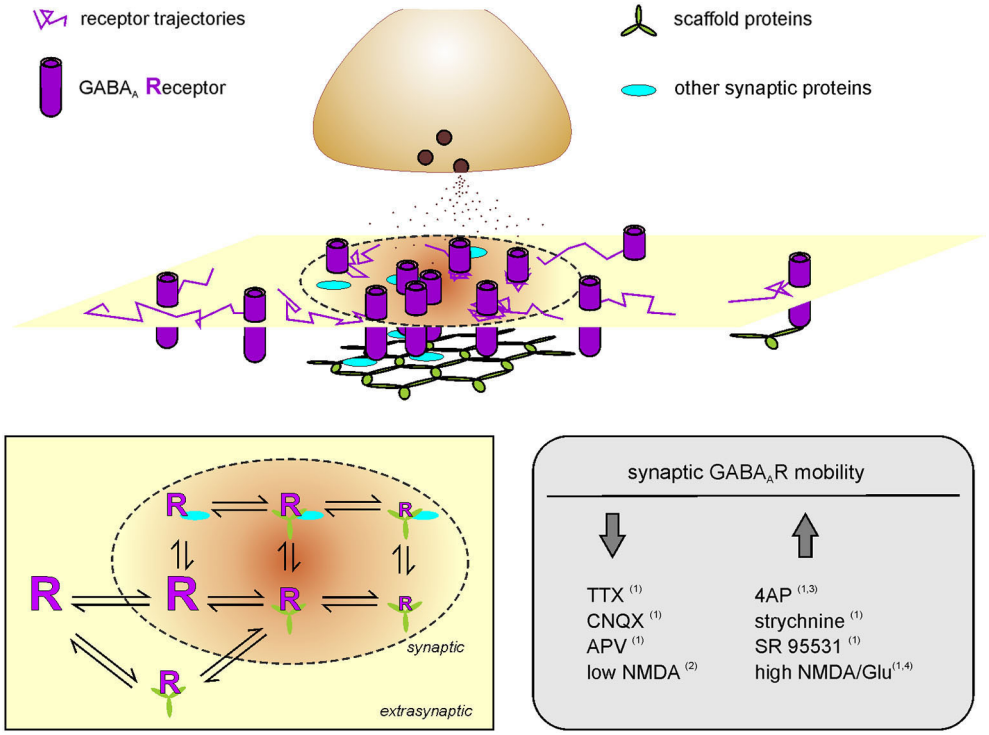

FIGURE 1 | Activity-dependent modulation of GABA$_A$ receptor diffusion trapping. Schematic representation of a postsynaptic membrane where GABA$_A$Rs laterally diffuse. Receptor trajectories are more confined at the inhibitory PSD (dotted line) as compared to extrasynaptic compartments. The reduced diffusion and transient stabilization of GABA$_A$Rs at the inhibitory synapse is favored by receptor interactions with scaffold proteins and by the presence of other synaptic proteins that provide molecular obstacles to receptor dynamics. Receptor scaffold interactions can also occur at extrasynaptic areas. Left inset: Diffusion-reaction model of GABA$_A$R surface mobility and interaction with stabilizing proteins. GABA$_A$Rs can freely diffuse in the neuronal membrane and exchange between synaptic and extrasynaptic compartments. At the inhibitory synapse, receptor interaction with scaffold proteins (green) and/or other postsynaptic proteins (cyan) can reduce and confine GABA$_A$R dynamics to various levels of stabilization. Receptor mobility is represented by the size of the letter "R". Some receptor-scaffold complexes can be formed also extrasynaptically and exchange with the synapse as a whole. Right inset right: Summary of the modulation of surface GABA$_A$R mobility at inhibitory synapses in the hippocampus, upon pharmacologically-induced changes of neuronal activity. Bannai et al. (2009), Muir et al. (2010), Niwa et al. (2012) and Petrini et al. (2014).

synaptic proteins through the regulation of their maturation/secretory pathway during basal activity and synaptic plasticity (Mabb and Ehlers, 2010; Vithlani et al., 2011; Lu and Roche, 2012). Ubiquitination consists in the covalent attachment of one or more copies of the 76-amino acid ubiquitin monomer to lysine residues of target proteins (Hurley and Stenmark, 2011). By serving as a sorting signal on protein cargo or by controlling the efficiency of the trafficking machinery, ubiquitination regulates protein transport between membrane compartments, hence playing a key role in the modulation of synaptic efficacy (Lin and Man, 2013). The contribution of activity-dependent receptor ubiquitination, specifically of polyubiquitination, in the translocation of proteins from the endoplasmic-reticulum (ER) has been mainly assessed for GABA$_A$Rs. Indeed, increased GABA$_A$R ubiquitination on β3 subunit upon chronic blockade of neuronal activity redirects newly assembled receptor from the ER back into the cytosol for subsequent proteasomal degradation, thus reducing GABA$_A$R synaptic accumulation and decreasing synaptic inhibition (Saliba et al., 2007). Similarly, massive activation of L-type voltage-gated calcium channels (VGCCs) depresses the efficacy of inhibitory synaptic transmission by negatively controlling receptor turnover and membrane stability with the ubiquitin-proteasome system (Saliba et al., 2009). On the contrary, increased neuronal activity decreases GABA$_A$R ubiquitination and enhances receptor stability in the plasma membrane (Saliba et al., 2007). Those pieces of evidence suggest that the ubiquitin-dependent proteasomal degradation is involved in a bidirectional adaptive modulation of surface receptor number. The ubiquitination can also trigger GABA$_A$R degradation via the lysosomal pathway, when this post-translational modification involves a motif within the intracellular domain of the γ2 subunit (Arancibia-Cárcamo et al., 2009).

Another receptor post-translational modification is palmitoylation, a reversible lipid modification occurring at the intracellular domain of neurotransmitter receptors—such as GABA$_A$R and AMPA receptors (Rathenberg et al., 2004; Hayashi et al., 2005), scaffold proteins (El-Husseini Ael et al., 2002; Dejanovic et al., 2014), and other receptor interacting proteins (Hanley and Henley, 2010). The palmitoylation of GABA$_A$R γ2 subunit has been demonstrated to favor the assembly

and clustering of $GABA_A$Rs by promoting their translocation through the Golgi apparatus to the neuronal membrane (Keller et al., 2004; Rathenberg et al., 2004; Fang et al., 2006). Impairment of the Golgi-specific palmitoyl acyltransferase GODZ, that mediates the palmitoylation of $GABA_A$R γ2 subunit, selectively reduces $GABA_A$Rs at synapse, thus decreasing the amplitude of inhibitory synaptic currents (Fang et al., 2006).

ADDITIONAL MECHANISMS FOR THE POSTSYNAPTIC CONTROL OF INHIBITORY SYNAPTIC STRENGTH

As introduced above, the strength of inhibitory signals can be also tuned by the intracellular chloride gradient that, in turn, depends on chloride transporters (Payne et al., 2003). It has been reported that the local decrease of K-Cl-cotransporter 2 (KCC2) efficiency reduces the strength of inhibition (Woodin et al., 2003). Even more importantly, the cotransport function is susceptible to changes in network activity. That is, increased glutamatergic transmission affects the chloride transport through the regulation of KCC2 phosphorylation at Ser 940 residue by PKC and PP1, which consecutively modulates KCC2 membrane trafficking (Rinehart et al., 2009; Lee et al., 2011). As a result of such altered chloride concentration, the activity-dependent regulation of IPSCs reversal potential directly shapes synaptic transmission (Wang et al., 2006; Saraga et al., 2008). Recently, it has also been demonstrated that the intracellular chloride concentration can determine the postsynaptic expression of $GABA_A$R α1, α3 and δ subunits, resulting in altered inhibitory synaptic transmission (Succol et al., 2012). Given the importance of these $GABA_A$R subunits in controlling the strength of phasic and tonic GABAergic activity, such changes in $GABA_A$R subunit expression are expected to strongly impact inhibitory network functioning (Brickley et al., 1996; Mozrzymas et al., 2007; Brickley and Mody, 2012).

Gene expression and mRNA translation of receptor subunits can further modulate the surface availability and synaptic accumulation of neurotransmitter receptors during synaptic plasticity (Mameli et al., 2007; Jung et al., 2014). For instance, the upregulation of $GABA_A$Rs and gephyrin proteins contributes to iLTP expression, while their downregulation has been observed during status epilepticus in the CA1 region of the hippocampus (Peng et al., 2004; González et al., 2013; Petrini et al., 2014). Moreover, it has been reported that fear conditioning regulates the gene expression of gephyrin in the amygdala (Ressler et al., 2002; Chhatwal et al., 2005). It is worth mentioning that mRNA encoding for proteins involved in the same plasticity process are co-assembled into the same RNA granules and targeted to dendrites (Gao et al., 2008). The coordination of such multiplexed dendritic targeting of different RNAs implies an adequate regulation to orchestrate gene expression at the synapse in the most metabolically and temporally efficient way.

DIFFUSION TRAPPING OF RECEPTOR LATERAL MOBILITY INFLUENCES SYNAPTIC SIGNALING

The early 2000s have witnessed a revolution in the notion of neurotransmitter receptor trafficking, when the direct observation of individual receptor lateral diffusion in the plane of the neuronal membrane was documented (Meier et al., 2001; Borgdorff and Choquet, 2002; Tovar and Westbrook, 2002; Dahan et al., 2003; Meissner and Haberlein, 2003; Tardin et al., 2003; Thomas et al., 2005; Burli et al., 2010; Fernandes et al., 2010). As such, the concept of the synapse has turned from a static entity endowed with semi-permanent neurotransmitter receptors to a dynamic structure where the number, type and position of receptors constantly change by lateral diffusion. The lateral mobility of surface neurotransmitter receptors in the neuronal membrane consists of thermally-driven Brownian movements susceptible to reversible stop-and-goes due to the interactions of the receptors with stable anchoring proteins (acting as "diffusion traps"—mainly at synapses) and to the molecular crowding of nonspecific obstacles (the "pickets and fences") (**Figure 1**). Therefore, the surface diffusion of neurotransmitter receptors is influenced by the protein and lipid composition of the receptor microenvironment, resulting in highly heterogeneous surface dynamics (Owen et al., 2009; Renner et al., 2009). Free receptor diffusion, with typical diffusion coefficient values of 0.1–1 $\mu m^2/s$, is mainly observed at extrasynaptic compartments, whereas in specialized areas such as synapses, receptor diffusion coefficients can reach even three to four orders of magnitude smaller values, largely due to diffusion traps (Calamai et al., 2009; Petrini et al., 2009; Muir et al., 2010). Indeed, both at inhibitory and excitatory synapses, the interaction of neurotransmitter receptors with scaffold proteins represents the major cause of receptor transient corralling in the postsynaptic area, as in the case of $GABA_A$R-gephyrin (Jacob et al., 2005), GlyR-gephyrin (Meier et al., 2001), AMPAR-PSD95-stargazin (Bats et al., 2007), mGluR-Homer (Sergé et al., 2002), D1 receptors-SAP102 (Thurner et al., 2014).

Receptor diffusion trapping at inhibitory synapses was first demonstrated in spinal cord neurons by the temporary stabilization and confinement of Glycine receptors (GlyRs) at synapses by the interaction of the receptor β subunit with gephyrin (Meier et al., 2001). Recently, Masson and colleagues have proposed that gephyrin acts as an energy trap for GlyRs, with a depth modulated by the biochemical properties of the receptor-gephyrin interaction domain (Masson et al., 2014). Gephyrin is also a scaffold protein for $GABA_A$Rs. The binding of the α1 subunit intracellular TM3-4 loop with gephyrin E domain reduces the diffusion, mediates the accumulation and increases the dwell time of $GABA_A$R at gephyrin-positive synaptic sites, thus concomitantly tuning the strength of synaptic inhibition (Mukherjee et al., 2011). In line with this, also the documented interaction of α2, α3, β2–3 and γ2 subunits of $GABA_A$R with gephyrin sets the physical condition for the aforementioned diffusion trapping of $GABA_A$R at synapses (Tretter et al., 2008; Maric et al., 2011; Mukherjee et al., 2011; Kowalczyk et al., 2013; Mou et al., 2013). It is worth noting that the major subunits composing extrasynaptic $GABA_A$Rs lack binding sites for gephyrin, thus accounting for the exclusion of α1/4/6α5βγ receptors from synapses (Wu et al., 2012). During potentiation of tonic inhibition, when the surface delivery of α4 and α5 containing $GABA_A$Rs is increased, the limited possibilities of these newly exocytosed receptors to be stabilized at synapses would favor their

accumulation at extrasynaptic sites, despite they might transiently explore inhibitory synapses by lateral diffusion (Renner et al., 2012; Saliba et al., 2012; Bright and Smart, 2013; Abramian et al., 2014). Although, the α5 subunit of $GABA_A$Rs has been mainly observed at extrasynaptic sites, some evidence indicates that it can also localize at GABAergic synapses (Brünig et al., 2002; Wu et al., 2012). It has been proposed that the synaptic localization of α5 subunits might be indirectly mediated by the anchoring of other synaptic $GABA_A$R subunits assembled within the same receptor (Brünig et al., 2002; Wu et al., 2012; Gerrow and Triller, 2014). Recently, the lateral mobility and synaptic accumulation of α2 and α5 subunit-containing $GABA_A$Rs has been reported to be oppositely modulated by $GABA_B$ receptor, likely due to the competition between these two $GABA_A$R subtypes for binding slots on synaptic scaffold proteins (Gerrow and Triller, 2014). Therefore, the preferential localization and diffusion trapping of distinct $GABA_A$R subunits at synaptic or extrasynaptic compartments would be governed by the subunit assortment of $GABA_A$Rs and by the affinity of receptor-scaffold interactions. In line with this, the diverse affinities of α1, α2 and α3 subunits for gephyrin can confer different diffusion properties to the mobility of synaptic $GABA_A$Rs (Maric et al., 2011). Of note, it has been recently quantified that dimeric inhibitory receptor fragments bind dimeric gephyrin with a ∼25-fold enhanced affinity compared to their monovalent counterparts (Maric et al., 2014). Considering that the typical synaptic αβγ $GABA_A$Rs bear 4 potential gephyrin-binding domains (two on α and two on β subunits) (Tretter et al., 2008, 2011; Mukherjee et al., 2011; Kowalczyk et al., 2013) and that gephyrin oligomerization can range from dimers to dodecamers (Linsalata et al., 2014), the multivalency of receptor-scaffold interaction represents an additional key regulator of inhibitory receptor stabilization at synapses.

Phosphorylation events can further modulate the affinity of the receptor-scaffold interactions, resulting in changes in synaptic efficacy. For instance, a phosphomimetic mutation of Thr 347 on the gephyrin-interacting domain of the $GABA_A$R α1 subunit reduces the affinity of $GABA_A$R-gephyrin binding, decreases receptor trapping at synapses and depresses inhibitory synaptic transmission (Mukherjee et al., 2011). Similarly, a PKC-mediated phosphorylation of Ser 403 within the cytoplasmic domain of the β-subunit of the GlyR increases receptor lateral mobility at synapses by reducing the binding affinity between GlyR intracellular loop and gephyrin, thus contributing to the plasticity of inhibitory synapses (Specht et al., 2011). Also the phosphorylation of gephyrin can tune the strength of the receptor tethering (Zacchi et al., 2014). It has been demonstrated by mass spectrometry that gephyrin harbors 22 phosphorylation sites which can influence gephyrin folding and clustering (Herweg and Schwarz, 2012; Kuhse et al., 2012; Tyagarajan et al., 2013; Tyagarajan and Fritschy, 2014). In particular gephyrin phosphorylation of Ser 270 by GSK3β or CDK5 and of Ser 268 by ERK negatively regulates the clustering of gephyrin and $GABA_A$Rs at synapses, threfore affecting inhibitory synaptic transmission (Kuhse et al., 2012; Tyagarajan et al., 2013; Tyagarajan and Fritschy, 2014). Furthermore, the conformational change of gephyrin induced by phosphorylation-dependent prolyl isomerase (Pin1) increases the stability of the scaffold lattice and the strength of GlyRs anchoring (Zita et al., 2007).

The regulation of receptor diffusion trapping at inhibitory synapses exhibits an additional level of complexity represented by the receptor-scaffold interactions occurring at extrasynaptic areas (Ehrensperger et al., 2007; Calamai et al., 2009). The dynamic equilibrium of synaptic and extrasynaptic receptor/scaffold interactions has been satisfactorily described by several computational models assuming that: (i) receptor-scaffold complexes can be formed outside and inside synapses; (ii) both preformed receptor-scaffold complexes and receptors alone can enter and leave the synapse; and (iii) the interaction of the receptors with the postsynaptic scaffold mainly accounts for receptor stabilization within the synapse (Ehrensperger et al., 2007; Calamai et al., 2009; Gerrow and Triller, 2010; Haselwandter et al., 2011; **Figure 1**, left inset). In this context, it has been additionally postulated that the size of synaptic clusters is maintained by a dynamic equilibrium between scaffold-scaffold aggregating forces and receptor-receptor repulsions (Haselwandter et al., 2011). Moreover, it cannot be excluded that other protein-protein interactions among molecular components of the synapse, including other structural proteins within the PSD, can contribute to receptor (and/or receptor-scaffold) trapping (**Figure 1**, left inset).

A more comprehensive picture of receptor diffusion trapping should also take into account endocytic zones (EZs) as specialized compartments where receptors mobility can be transiently reduced and confined, similarly to synapses. It has been demonstrated that $GABA_A$R β3 and AMPAR GluA2 subunits can interact with AP2 (Lee et al., 2002; Kittler et al., 2008; Smith et al., 2012), leading to a reversible trapping of surface $GABA_A$ and glutamate receptors at EZs, respectively (Petrini et al., 2009; Smith et al., 2012). Indeed, after a temporary retention at EZs, $GABA_A$ and AMPA receptors recover free lateral diffusion upon exit from these specialized areas. Therefore, in addition to receptor removal from the surface, EZs contribute to the regulation of synaptic receptor number by transiently retaining the mobility of surface receptors.

ACTIVITY-DRIVEN MODULATION OF $GABA_A$ RECEPTOR DYNAMICS FOR THE EXPRESSION OF SYNAPTIC PLASTICITY

Over the last decade, compelling evidence has documented that receptor lateral diffusion can be modulated in response to changes of neuronal activity (**Figure 1**, right inset). It has been reported that the impairment of synaptic activity by preventing action potential firing with the sodium channel blocker tetrodotoxin (TTX) significantly reduces the lateral diffusion of $GABA_A$Rs in hippocampal cells (Bannai et al., 2009), although this effect has not been observed in spinal cord neurons (Lévi et al., 2008). In the hippocampus, a similar $GABA_A$R immobilization was induced by blocking glutamatergic transmission with CNQX and APV (Bannai et al., 2009). Conversely, the lateral mobility of $GABA_A$Rs increased upon induction of neuronal hyperactivity either by blocking potassium channels with 4-aminopyridine (4AP) or by dampening inhibition with GlyR and $GABA_A$R antagonists

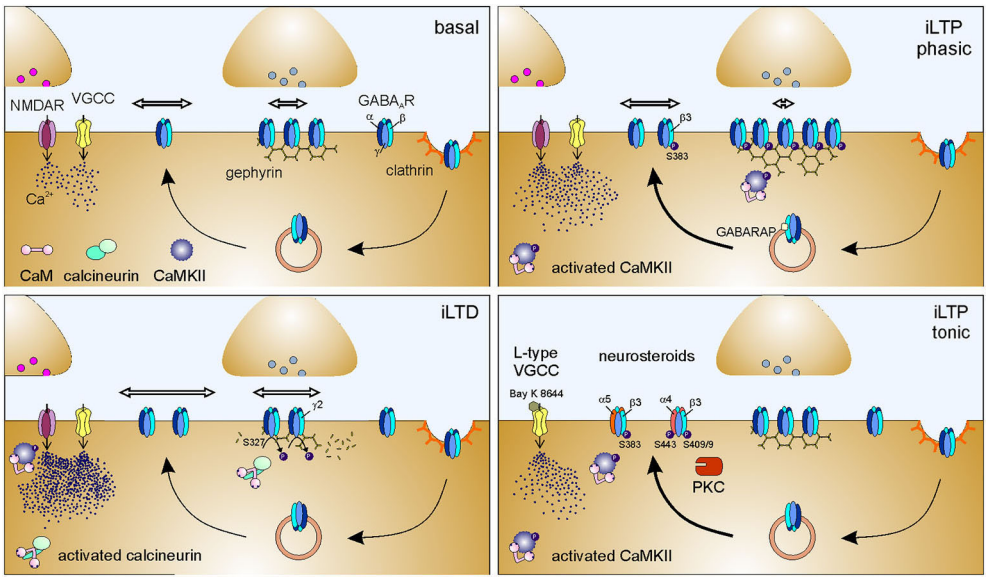

FIGURE 2 | Molecular mechanisms of postsynaptic plasticity of inhibitory synapses. Basal: Simplified sketch of the molecular components of the inhibitory synapse in basal conditions. For schematization purposes, only the scaffold protein gephyrin and $GABA_A$Rs are represented at the inhibitory PSD. Line arrows indicate $GABA_A$R intracellular trafficking, namely exocytosis, clathrin-mediated endocytosis and recycling. Thicker line arrows indicate potentiated trafficking. Horizontal hollow arrows indicate surface $GABA_A$R lateral mobility; the arrow length is proportional to receptor surface diffusion. Modifications of intracellular Ca^{2+} concentrations can be mediated by the activation of NMDA receptors (NMDARs) and/or voltage-gated calcium channels (VGCCs). The molecular changes occurring during iLTD and iLTP, schematized in the other panels, should be compared to the conditions represented here. iLTD: Postsynaptically-expressed inhibitory long-term depression (iLTD) is triggered by massive intracellular Ca^{2+} increase (mediated by NMDA receptors and/or VGCCs), that leads to the activation and recruitment of calcineurin to inhibitory synapses. Calcineurin mediates the dephosphorylation of Ser 327 on $GABA_A$R γ2 subunit which in turn increases the lateral mobility of synaptic and extrasynaptic $GABA_A$Rs, thus promoting the dispersion of synaptic receptors. Hence, inhibitory synapses exhibit a reduced number of $GABA_A$Rs, resulting in decreased inhibitory synaptic strength. iLTD correlates with a reduction of gephyrin clustering at synapses (Lu et al., 2000; Wang et al., 2003a; Bannai et al., 2009; Muir et al., 2010; Niwa et al., 2012). iLTP phasic: Postsynaptically-expressed long-term potentiation of inhibitory synaptic currents (iLTP phasic) is elicited by NMDA-induced moderate increase of intracellular Ca^{2+} that recruits activated CaMKII to inhibitory synapse and promotes GABARAP-mediated $GABA_A$R exocytosis. It cannot be excluded that VGCCs are contributing to the Ca^{2+} increase leading to iLTP. CaMKII phosphorylates at least Ser 383 on $GABA_A$R β3 subunit, an event that enhances the accumulation of gephyrin at the postsynaptic level and selectively promotes the immobilization of synaptic $GABA_A$Rs, while leaving the lateral diffusion of extrasynaptic receptors unaltered. As a result, inhibitory synapses are endowed with a larger number of $GABA_A$Rs that accounts for increased inhibitory synaptic strength (Marsden et al., 2007; Petrini et al., 2014). iLTP tonic: Long-term potentiation of tonic inhibition (iLTP tonic) is mediated by the activation of L-type VGCCs (demonstrated by the sensitivity to dihydropyridine Bay K 8644, which stabilizes the channel open state). The consequent moderate increase of intracellular Ca^{2+} promotes the CaMKII-mediated phosphorylation of Ser 383 on $GABA_A$R β3 subunit that in turn enhances the exocytosis of α5-containing $GABA_A$Rs. This results in an increased number of surface α5-containing $GABA_A$Rs, that, being predominantly extrasynaptic, potentiate tonic inhibitory currents (Saliba et al., 2012). When tonic iLTP is promoted by neurosteroids, the activation of PKC leads to the phosphorylation of Ser 443 on α4 subunit and on Ser 408/409 on β3 subunit of $GABA_A$R. These events enhance the exocytosis and the membrane stability of α4 subunit-containing receptors, resulting in potentiated tonic currents (Abramian et al., 2010, 2014 but see also Bright and Smart, 2013).

(Bannai et al., 2009; Niwa et al., 2012), thus suggesting a positive correlation between $GABA_A$R lateral diffusion and neuronal activity (**Figure 1**, right inset). The bidirectional modulation of receptor lateral mobility by neuronal activity provides a further control of receptor number at synapses during synaptic plasticity. It should be emphasized that this mechanism operates in the tens-of-milliseconds time range, being considerably faster that receptor recycling (Choquet and Triller, 2013). The role of receptor lateral mobility in the expression of long-term synaptic plasticity has been addressed in several studies at both glutamatergic and GABAergic synapses (Bannai et al., 2009; Makino and Malinow, 2009; Petrini et al., 2009, 2014; Muir et al., 2010). For instance, it has been recently demonstrated that the postsynaptic potentiation of inhibition, chemically induced in cultured hippocampal neurons by moderate NMDAR activation, relies on the enhanced accumulation and immobilization of surface $GABA_A$Rs at synapses (Petrini et al., 2014; **Figure 2**). Such immobilization of synaptic $GABA_A$Rs during iLTP has been explained by the promoted clustering of gephyrin at synapses. It has been proposed that the NMDA-induced moderate intracellular Ca^{2+} rise that triggers iLTP increases the phosphorylation of Ser 383 on $GABA_A$R β3 subunit by CaMKII (Petrini et al., 2014). This event is crucial to promote the surface delivery of $GABA_A$Rs and the recruitment of gephyrin to inhibitory synapses (Marsden

et al., 2007; Petrini et al., 2014). Following the general rule mentioned above—concerning the influence of scaffold availability on receptor diffusion trapping—the increased clustering of gephyrin at the inhibitory PSD during iLTP promotes the corralling and prolongs the residence time of synaptic $GABA_A$Rs, thus increasing inhibitory synaptic strength (Petrini et al., 2014). Of note, by selectively tracking the mobility of surface $GABA_A$Rs before and after iLTP induction, Petrini et al. (2014) demonstrated that also preexisting surface $GABA_A$Rs are recruited and immobilized at synapses during potentiation of inhibition. This observation describes an additional source of $GABA_A$Rs accumulated at synapses during iLTP, besides the previously known promoted CaMKII-dependent exocytosis of $GABA_A$Rs (Marsden et al., 2007; **Figure 2**).

It has been recently reported that an analogous mechanism based on postsynaptic modifications of GABAergic synapses underlies a form of potentiation of inhibition *in vivo*. Indeed, two independent studies have addressed the molecular determinants of iLTP observed *in vivo* in principal cells of the layer IV of rat visual cortex (Nahmani and Turrigiano, 2014; Petrini et al., 2014) after a brief protocol of monocular deprivation (MD) at the peak of the critical period (Maffei et al., 2006). With immunoelectron microscopy and confocal imaging, both studies have demonstrated that postsynaptic $GABA_A$Rs are more enriched in MD animals expressing iLTP as compared to controls (Nahmani and Turrigiano, 2014; Petrini et al., 2014). Moreover, Petrini et al. (2014) have also documented an increase of gephyrin clusters immunoreactivity after the MD protocol. Overall those pieces of evidence indicate that a coordinated control of $GABA_A$R and gephyrin dynamics could be a general mechanism underlying the postsynaptic expression of inhibitory synaptic potentiation *in vitro* and *in vivo*, at least in the rat visual cortex.

An opposite regulation of $GABA_A$R and gephyrin with respect to that observed during iLTP has been described during iLTD induced by (i) trains of depolarizing stimuli; (ii) pharmacologically-induced increased excitatory neuronal activity (4AP); or (ii) strong NMDA receptor activation (Lu et al., 2000; Bannai et al., 2009; Muir et al., 2010; Niwa et al., 2012). In particular, at molecular level, the depression of synaptic inhibition relies on the recruitment of activated calcineurin at inhibitory synapses and on the declustering of gephyrin, along with the dispersal of synaptic $GABA_A$Rs by augmented lateral diffusion (Bannai et al., 2009; Marsden et al., 2010; Muir et al., 2010; **Figure 2**). The increased lateral mobility of surface $GABA_A$R observed during iLTD (induced by 4AP or NMDA) correlates with reduced $GABA_A$R synaptic dwell time and increased confinement area through the calcineurin-mediated dephosphorylation of Ser 327 of the γ2 subunit (Marsden et al., 2010; Muir et al., 2010). However, the involvement of calcineurin in mediating gephyrin synaptic decrease during iLTD depends on the stimulus applied. Indeed, after 4AP stimulation, calcineurin increases $GABA_A$R lateral mobility and reduces both $GABA_A$R and gephyrin clusters, whereas after NMDA application it only controls $GABA_A$R cluster size (Niwa et al., 2012). This suggests that the regulation of gephyrin clustering during NMDA-dependent iLTD does not depend on calcineurin activity. The data discussed so far indicate that moderate or high intracellular Ca^{2+} rise oppositely affects $GABA_A$R surface dynamics leading to either iLTP or iLTD, respectively (Bannai et al., 2009; Marsden et al., 2010; Muir et al., 2010; Petrini et al., 2014). This might be explained in terms of the regulated recruitment of CaMKII at inhibitory synapses. In this concern, Marsden et al. (2010) have proposed that low Ca^{2+} recruits activated CaMKII at inhibitory synapses, while high Ca^{2+} leads to calcineurin localization at GABAergic synapses, where it prevents the accumulation of CaMKII (**Figure 2**). It is worth discussing that, the plasticity of excitatory synapses follows an opposite Ca^{2+} rule with respect to that of inhibitory synapses, as low and high Ca^{2+} trigger LTD and LTP, respectively (Lee et al., 2003; He et al., 2011). Therefore, the spatiotemporal dynamics of Ca^{2+} concentration can determine the bidirectional plasticity of both excitatory and inhibitory synapses. The coordination of these convergent Ca^{2+} signaling pathways is expected to be a main determinant for the fine control of the excitation/inhibition balance (E/I).

Besides the long-term synaptic plasticity, lateral diffusion has also been described to be crucial for the short-term plasticity at glutamatergic synapses. During rapid repetitive synapse activation, lateral diffusion allows desensitized AMPA receptors to leave the synapse and to be replaced with mobile extrasynaptic naïve receptors in tens of milliseconds, thus favoring the recovery from high-frequency synaptic depression (Heine et al., 2008). Those pieces of evidence suggest that extrasynaptic receptors constitute a reservoir pool of receptors which may exchange with desensitized synaptic receptors, thereby representing a gear for controlling the fidelity of synaptic transmission during high-frequency synaptic activation. In this regard, the kinetics of receptor desensitization would set the time window in which surface receptor dynamics can contribute to synaptic strength. At GABAergic synapses, the impact of surface $GABA_A$R dynamics on short-term synaptic plasticity has not been addressed yet. Nevertheless, the fact that $GABA_A$Rs desensitized state(s) can live for milliseconds-to-seconds periods (Jones and Westbrook, 1995; Petrini et al., 2011) suggests that desensitized $GABA_A$Rs might exchange between synaptic and extrasynaptic compartments, providing the theoretical background for a multi-scaled temporal regulation of $GABA_A$R dynamics on the fidelity of high-frequency inhibitory synaptic transmission. Future investigations will be required to clarify how $GABA_A$R gating and lateral mobility cooperate to achieve the fine tuning of synaptic strength as a function of the frequency of synaptic activity.

DYNAMICS OF OTHER SYNAPTIC PLAYERS DURING INHIBITORY SYNAPTIC PLASTICITY

In addition to neurotransmitter receptors, other proteins of the PSD represent key players of synaptic function, including scaffold and adhesion proteins, as well as structural elements, such as cytoskeleton and microtubules (Gordon-Weeks and Fournier, 2014; Tyagarajan and Fritschy, 2014). Noteworthy, synaptic scaffold proteins can laterally diffuse at submembrane level and even more importantly, their diffusive properties can be regulated in activity-dependent manner (Hanus et al., 2006; Sharma et al., 2006). Hence, the expression of synaptic plasticity implies

dynamic and efficient adjustments not only of neurotransmitter receptor number at synapses, but of the whole postsynaptic structure and composition. For instance, the enrichment of synaptic gephyrin described in Petrini et al. (2014) as a key step for iLTP expression is initially sustained by the recruitment of extrasynaptic gephyrin to synapses. Of note, despite the increase of synaptic gephyrin is necessary for the potentiation of inhibition, gephyrin redistribution to synapses does not precede the accumulation of synaptic $GABA_A$Rs. This evidence challenges the traditional notion that unbound receptors can be exclusively tethered at free docking slots available at synapse and it points towards the concept that the dynamics of scaffold molecules occurs in concert with that of neurotransmitter receptors both at synaptic and extrasynaptic compartments. The idea that during synaptic plasticity changes in scaffold dynamics precede neurotransmitter receptor rearrangements has been similarly challenged by Niwa and coworkers. The authors demonstrate that, during iLTD, the dispersal of synaptic $GABA_A$R precede the reduction of gephyrin cluster size (Niwa et al., 2012). Despite, the role of scaffold dynamics in the reorganization occurring at the synapse during inhibitory synaptic plasticity starts to be unveiled with respect to gephyrin (Niwa et al., 2012; Petrini et al., 2014), future investigations of this issue should be broaden to include other anchoring/structural proteins of the inhibitory PSD.

Numerous pieces of evidence indicate a tight cross-regulation of many proteins of the inhibitory PSD. For instance, the impairment of collybistin dampens gephyrin and $GABA_A$R clustering (Jedlicka et al., 2009; Poulopoulos et al., 2009), the lack of dystrophin or of gephyrin reduces the stabilization of $GABA_A$R in a subset of synapses (Kneussel et al., 1999; Knuesel et al., 1999; Yu et al., 2007), but also the knock-out of $GABA_A$Rs subunits prevents the correct clustering of gephyrin (Essrich et al., 1998; Schweizer et al., 2003; Studer et al., 2006). Altogether this suggests that, theoretically, all synaptic elements can influence the localization and dynamics the other synaptic molecules (Specht and Triller, 2008). In this framework, it has been demonstrated that the adhesion proteins β1 and β3 integrin influence GlyR dwell time and gephyrin exchange at synapses, leading to altered inhibitory synaptic strength in the spinal cord (Charrier et al., 2010).

The mobility of gephyrin molecules has been distinguished in a fast and low component, namely rapidly oscillations with sub-micrometric lateral motion around their initial position with diffusion coefficients 10–20 times slower than $GABA_A$Rs and slow non-stochastic movements of entire gephyrin clusters over minutes-to-hours periods (Hanus et al., 2006; Maas et al., 2006; Calamai et al., 2009; Dobie and Craig, 2011; Kuriu et al., 2012). Both components of gephyrin dynamics are dependent on the presence of the cytoskeleton and microtubules (Hanus et al., 2006). The disruption of F-actin slows down rapid rearrangements and slower lateral displacements of gephyrin, whereas the impairment of microtubules only increases the slow lateral dynamics of gephyrin clusters (Hanus et al., 2006). Moreover, synaptic gephyrin can be additionally modulated by neuronal activity (Bausen et al., 2006; Hanus et al., 2006). This suggests that the reduction of rapid gephyrin dynamics observed during increased synaptic activity (Hanus et al., 2006) may depend on activity-dependent adjustments of the cytoskeleton and intracellular Ca^{2+} levels, likely influencing receptors stabilization at synapses (Wei et al., 2004). Another synaptic molecule reported to laterally diffuse in the neuronal membrane is the adhesion protein neuroligin1 (NLG1; Giannone et al., 2013). Despite NLG1 is mostly expressed at excitatory synapses, it has been also found at inhibitory synapses interacting with gephyrin (Levinson et al., 2005; Varley et al., 2011). The dynamic behavior of surface NLG1 is strongly reduced by presynaptic neurexin (Nrx). The binding to Nrx-1β favors the interaction of NLG1 with the intracellular excitatory scaffold and confers confined mobility to NLG1 (Giannone et al., 2013). Moreover, the phosphorylation state of Tyr 782 on NLG1 regulates the preferential binding of NLG1 to PSD95 or gephyrin, thus determining the different location of NLG1 at excitatory or inhibitory synapses (Giannone et al., 2013). Considering those data, it can be hypothesized that the activity-induced phosphorylation of NLG1 would potentially define the enrichment of NLG1 at excitatory or inhibitory synapses through the regulation of NLG1 lateral diffusion. Recent evidence has documented that also the KCC2 transporter explores the postsynaptic membrane by lateral diffusion and is transiently stabilized both at excitatory and inhibitory synapses (Chamma et al., 2013). Importantly, KCC2 surface dynamics is activity-regulated, as indicated by the higher lateral mobility and reduced dwell time of KCC2 observed upon increased network activity. This dispersal of KCC2 is dependent on the dephosphorylation of Ser 940 (Chamma et al., 2013). Of note, the dephosphorylation of Ser 940 additionally accounts for the reduced chloride export and diminished intensity of hyperpolarizing GABAergic inhibition induced by NMDA receptor activation (Lee et al., 2011), therefore suggesting that the activity-regulated dynamics of KCC2 can tune the strength of synaptic inhibition.

INTRASYNAPTIC NANOSCALED RECEPTOR DYNAMICS

The advent of super-resolution techniques and the progresses in EM tomography have allowed dissection of the structure and composition of the PSD at the single molecule level with nanometer resolution, revealing its subsynaptic clustered organization. Indeed, both at excitatory and inhibitory synapses, scaffold elements and receptors are arranged in nanostructures $<$100 nm wide (Chen et al., 2011; Fukata et al., 2013; MacGillavry et al., 2013; Nair et al., 2013; Specht et al., 2013). Although, as mentioned above, the postsynaptic scaffold undergoes constant molecular renewal and exhibits some dynamic rearrangements within the synapse, the subsynaptic scaffold nanodomains primarily represent stable hotspots for neurotransmitter receptor confinement (Sharma et al., 2006; Nair et al., 2013; Specht et al., 2013). Therefore, the idea of neurotransmitter receptors being highly mobile at extrasynaptic areas and stabilized at synapses by scaffold molecules requires a paradigm shift towards a more complex view in which the heterogeneity of the PSD composition and the nanodomains-based repartition of receptor mobility are taken into account (Gerrow and Triller, 2010; Choquet and Triller, 2013).

Importantly, such nanostructured organization of AMPA receptor dynamics has been reported to be activity-regulated,

thus indicating that the exploring behavior of AMPA receptors at synapses is tuned by synaptic activity. Local activity confines AMPA receptor dynamics in nanometer-sized intrasynaptic areas and reduces the diffusive exchange between synaptic and extrasynaptic compartments. On the contrary, at inactive synapses, AMPA receptors fully explore the PSD and are less efficiently retained at synapses (Ehlers et al., 2007). Interestingly, mild glutamate receptor activation is sufficient to increase the intrasynaptic lateral mobility of AMPA receptors, as observed upon partial photobleaching of the PSD (Kerr and Blanpied, 2012). Most of the knowledge of the nanoscaled dynamics of synaptic components has been achieved at excitatory synapses with the benefit from the conceptual and technical advancement of different groups (Giannone et al., 2010; Hoze et al., 2012; Kerr and Blanpied, 2012; MacGillavry et al., 2013; Nair et al., 2013; Lu et al., 2014). Recently, superresolution techniques have been also exploited in the study of inhibitory synapses, describing for the first time the correspondence between the intrasynaptic spatial distribution of gephyrin and GlyR at spinal cord neurons (Specht et al., 2013). Furthermore, by assessing the stoichiometry of gephyrin-receptor binding, the authors studied the activity-dependence of gephyrin cluster occupancy by GlyR and $GABA_AR$. They disclosed that, in spinal cord neurons, the blockade of spontaneous activity mostly affects the subset of inhibitory PSDs endowed with the largest $GABA_AR$ occupancy and lowest GlyR occupancy (Specht et al., 2013). This study lays the groundwork to deeply investigate in future years the nanoscopic rearrangements of the composition, distribution and dynamics of the synaptic components underlying the plasticity of inhibitory synapses. Furthermore, the precise location of $GABA_AR$ in the synaptic disc in relation to the position of the releasing site will be also crucial to determine the neurotransmitter concentration profile "seen" by postsynaptic receptors, an important determinant of synaptic strength (Barberis et al., 2011; Petrini et al., 2011).

CONCLUSIONS

The correct functioning of the synapse relies on the balance between the stability of synaptic structures and the dynamics of its molecular components, a concept that is fundamental for basal synaptic transmission and for the activity-dependent tuning of synaptic strength. This Review highlights the importance of postsynaptic protein dynamics for the expression of plasticity at inhibitory synapses. A large body of evidence collected over the last 20 years has documented the highly coordinated, yet not fully elucidated, regulation of $GABA_AR$ intracellular trafficking (additionally modulated by receptor post-translational modifications), which controls surface receptor content to finally tune inhibitory synaptic efficacy. Remarkable technical and conceptual progresses achieved during the last decade have revealed that surface receptor lateral mobility is crucial to allow fast and persistent adjustments of ready-to-be-activated receptors at the synapse. Indeed, receptor lateral diffusion sustains (i) the constant renewal of synaptic receptors; and (ii) the activity-regulated dynamic redistribution of surface receptors to, within and from the synapse. The importance of postsynaptic protein dynamics in synaptic plasticity has been further reinforced when the notion of the postsynaptic scaffold has evolved from a "passive tether for synaptic receptors" towards a dynamic scenario in which receptor-scaffold interactions could be modulated over a wide range of time and intensities to ultimately regulate receptor diffusion trapping.

Despite our understanding of how activity-regulated protein dynamics contributes to inhibitory synaptic plasticity has significantly expanded, some of the underlying mechanisms remain fragmentary. In addition to the knowledge gaps highlighted throughout this Review, the comprehension of inhibitory postsynaptic plasticity will require to fully elucidate the influence of the molecular heterogeneity of GABAergic synapses on the dynamic remodeling of the synaptic molecular components. In addition to gephyrin, many other scaffold proteins composing the inhibitory PSD (eventually expressed in different isoforms and splice variants), should be analyzed during activity dependent synaptic reorganization. In this regard, the preferential binding of $GABA_AR$ subtypes to selected anchoring proteins would provide an additional level of complexity in the study of the modulation of receptor-scaffold interactions in basal conditions and during synaptic plasticity. For instance, in perisomatic synapses of CA1 pyramidal cells, in a subset of inhibitory synapses in cortical neurons, in cerebellar Purkinje neurons, the $GABA_AR$ α1 subunit is preferentially associated with the dystrophin-glycoprotein complex, whereas the α2 subunit better interacts with gephyrin (Panzanelli et al., 2011). Furthermore, it is still unclear whether the assortment and the synapse-specific localization of inhibitory postsynaptic scaffold proteins selectively modulate the expression of plasticity at subsets of synapses. This issue should also be contextualized to the rich diversity of GABAergic interneurons impinging on principal cells in specific compartments of the somato-dendritic axis (Klausberger and Somogyi, 2008).

The deeper investigation of the intracellular biochemical pathways activated during the plasticity of inhibitory synapses will also be fundamental to elucidate which mechanisms are shared with the plasticity of excitatory synapses for the future understanding of the coordination of activity-dependent adjustments of excitatory and inhibitory synaptic strength. The relevance of this issue lies in the functional crosstalk between excitatory and inhibitory synapses. That is, activity-dependent changes at excitatory and inhibitory synapses will not only result in altered E/I balance but also, at network level, they can be differently integrated to ultimately promote Hebbian plasticity or homeostatic stabilization of neuronal network activity (Vitureira and Goda, 2013).

Several lines of research have recently attempted to characterize *in vivo* inhibitory synaptic plasticity induced by environmental and chemical stimuli in different brain areas. For instance, multiple forms of potentiation and depression of GABAergic signaling have been described *in vivo* in response to changes of the animal sensory experience (e.g., ocular dominance, stress, fear acquisition and extinction) and to exposure to drugs (e.g., cocaine, ethanol, neurosteroids) (Maffei et al., 2006; Gonzalez et al., 2012; Li et al., 2012; Bocklisch et al., 2013; Inoue et al., 2013). Some of the molecular modifications and the cell-signaling pathways involved in the inhibitory synaptic plasticity *in vivo* start to be unveiled, as in the case of dynamic regulation of surface $GABA_AR$ and gephyrin levels during fear memory consolidation

and after monocular deprivation (Chhatwal et al., 2005; Heldt and Ressler, 2007; Nahmani and Turrigiano, 2014; Petrini et al., 2014). However, there is increasing need to focus on protein dynamics *in vivo* for a more realistic description of the post-synaptic determinants underlying activity-dependent changes of inhibitory synaptic strength and their coordination. With the growing technological progress in neurophotonics, it is desirable to achieve *in vivo* the accuracy already obtained *in vitro* about the precise involvement of receptor and scaffold dynamics in the expression of inhibitory synaptic plasticity.

ACKNOWLEDGMENTS

This work has been supported by Telethon-Italy (GGP11043) to Andrea Barberis and Enrica Maria Petrini and Compagnia di San Paolo (ROL-4318) to Andrea Barberis.

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

Received: 12 August 2014; paper pending published: 27 August 2014; accepted: 05 September 2014; published online: 23 September 2014.

Citation: Petrini EM and Barberis A (2014) Diffusion dynamics of synaptic molecules during inhibitory postsynaptic plasticity. Front. Cell. Neurosci. ***8****:300. doi: 10.3389/fncel.2014.00300*

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

frontiers in
CELLULAR NEUROSCIENCE

ORIGINAL RESEARCH ARTICLE
published: 11 August 2014
doi: 10.3389/fncel.2014.00222

Ovarian cycle-linked plasticity of δ-$GABA_A$ receptor subunits in hippocampal interneurons affects γ oscillations *in vivo*

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$GABA_A$ receptors containing δ subunits (δ-$GABA_A$Rs) are GABA-gated ion channels with extra- and perisynaptic localization, strong sensitivity to neurosteroids (NS), and a high degree of plasticity. In selective brain regions they are expressed on specific principal cells and interneurons (INs), and generate a tonic conductance that controls neuronal excitability and oscillations. Plasticity of δ-$GABA_A$Rs in principal cells has been described during states of altered NS synthesis including acute stress, puberty, ovarian cycle, pregnancy and the postpartum period, with direct consequences on neuronal excitability and network dynamics. The defining network events implicated in cognitive function, memory formation and encoding are γ oscillations (30–120 Hz), a well-timed loop of excitation and inhibition between principal cells and PV-expressing INs (PV + INs). The δ-$GABA_A$Rs of INs can modify γ oscillations, and a lower expression of δ-$GABA_A$Rs on INs during pregnancy alters γ frequency recorded *in vitro*. The ovarian cycle is another physiological event with large fluctuations in NS levels and δ-$GABA_A$Rs. Stages of the cycle are paralleled by swings in memory performance, cognitive function, and mood in both humans and rodents. Here we show δ-$GABA_A$Rs changes during the mouse ovarian cycle in hippocampal cell types, with enhanced expression during diestrus in principal cells and specific INs. The plasticity of δ-$GABA_A$Rs on PV-INs decreases the magnitude of γ oscillations continuously recorded in area CA1 throughout several days *in vivo* during diestrus and increases it during estrus. Such recurring changes in γ magnitude were not observed in non-cycling wild-type (WT) females, cycling females lacking δ-$GABA_A$Rs only on PV-INs (PV-*Gabrd*$^{-/-}$), and in male mice during a time course equivalent to the ovarian cycle. Our findings may explain the impaired memory and cognitive performance experienced by women with premenstrual syndrome (PMS) or premenstrual dysphoric disorder (PMDD).

Keywords: ovarian cycle, PMS, PMDD, gamma oscillations, $GABA_A$ receptor, delta subunit, tonic inhibition, parvalbumin

INTRODUCTION

There are numerous reports about women experiencing fluctuating cognitive and neuropsychological functions during specific stages of the menstrual cycle. During the luteal phase, when progesterone levels are high, some women may experience different levels of dysthymia, irritability, anxiety, impaired working and emotional memory. All of these symptoms are inevitably aggravated in patients with premenstrual dysphoric disorder (PMDD; Man et al., 1999; Sveindóttir and Bäckstrøm, 2000; Bäckström et al., 2003; Reed et al., 2008; Ertman et al., 2011; Rapkin and Akopians, 2012; Yen et al., 2012; Bayer et al., 2014). Although all of these conditions can be hardly ascribed to a single mechanism, ovarian cycle-linked plasticity of δ-$GABA_A$Rs and resulting effects on tonic inhibition have been implicated in modifications in anxiety and memory performance in rodents (Maguire et al., 2005; Cushman et al., 2014). Moreover, patients with PMDD seem to be less sensitive to GABAergic modulation during their luteal phase, which led to hypothesize a luteal deficit of $GABA_A$Rs plasticity (Bäckström et al., 2003; Maguire et al., 2005).

The δ-$GABA_A$Rs are high affinity, low efficacy non-synaptic $GABA_A$ receptors with a high sensitivity to neurosteroids (NS; Brickley and Mody, 2012). During times of altered NS levels, δ-$GABA_A$Rs expression changes in a direction that seems to depend on the timing of NS fluctuations. For instance, δ-$GABA_A$Rs plasticity has been observed during the ovarian cycle, pregnancy, the postpartum, puberty and acute stress, with direct effects on neuronal excitability and network activity. In particular, δ-$GABA_A$Rs plasticity has been reported in both principal cells and INs in different rodent brain areas including the hippocampus, different nuclei of the thalamus and the periaqueductal gray (Lovick et al., 2005; Brack and Lovick, 2007; Maguire and Mody, 2007, 2008; Maguire et al., 2009; Ferando and Mody, 2013a; Smith, 2013; MacKenzie and Maguire, 2014).

The tonic conductance mediated by δ-$GABA_A$Rs constitutes a powerful constraint over gain of neuronal signal transmission in both principal cells and INs (Mody and Pearce, 2004; Semyanov et al., 2004; Farrant and Nusser, 2005; Walker and Semyanov, 2008; Song et al., 2011). The δ-$GABA_A$Rs of hippocampal INs

modulate γ oscillations frequency *in vitro* (Mann and Mody, 2010; Ferando and Mody, 2013a). The γ oscillations are a periodic network activity (30–120 Hz) that can be recorded in different brain areas during certain wakefulness states and REM sleep, and arise from a synchronized excitation and inhibition loop between principal cells and PV-INs, which have a critical role in initiating and maintaining local oscillations (Sohal et al., 2009; Wulff et al., 2009; Korotkova et al., 2010; Carlén et al., 2011; Zheng et al., 2011; Lasztoczi and Klausberger, 2014). These oscillations are thought to enable encoding and memory formation in discrete neuronal network, to facilitate spike-time dependent plasticity, and are considered to play an important role in the physiology of learning and memory (Colgin and Moser, 2010; Uhlhaas et al., 2011; Buzsáki and Wang, 2012; Uhlhaas and Singer, 2012). Because in this study we were interested in more gradual alterations in oscillatory activity (expected with the hormonal changes linked to the ovarian cycle) we focused on REM sleep which is characterized by prominent and regular θ – γ episodes, and is unaffected by instantaneously changing "external" parameters such as running speed (McFarland et al., 1975; Shin and Talnov, 2001; Ahmed and Mehta, 2012); instead, the θ – γ episodes during REM sleep rely on "internal" mechanisms such as emotional information processing and memory consolidation which are known to be affected by ovarian/menstrual cycle (Montgomery et al., 2008; Walker, 2009; Scheffzük et al., 2011).

In a recent study in mice we showed a homeostatic pregnancy-related down-regulation of δ-GABA$_A$Rs in CA3 stratum pyramidale (SP) INs which led to an increase in the frequency of γ oscillations recorded *in vitro* (Ferando and Mody, 2013a), in a manner similar to what has been observed in *Gabrd*$^{-/-}$ mice (Mann and Mody, 2010). However, the effects of δ-GABA$_A$R plasticity of INs on network activity and dynamics in the intact brain remain to be elucidated. In this study we show ovarian cycle-linked alterations in δ-GABA$_A$R expression in hippocampal CA1 and CA3 SP INs, on dentate gyrus granule cells (DGGCs) and pyramidal cells of the CA1, with increased expression during the high progesterone stage of diestrus, and decreased expression in estrus. These changes correlate with periodic modifications in the magnitude of γ oscillations recorded *in vivo* in CA1 SP of freely moving mice.

Previous studies showed increased anxiety and memory performance in female WT but not in global *Gabrd*$^{-/-}$ mice during estrus (low progesterone phase), while the behavior during diestrus in WT mice closely resembled that of male mice (Maguire et al., 2005; Moore et al., 2010; van Goethem et al., 2012; Cushman et al., 2014). However, δ-GABA$_A$Rs are plastic in both hippocampal principal cells and INs, so that behavioral correlates in global *Gabrd*$^{-/-}$ mice cannot distinguish between receptor changes in specific neuronal subtypes. By using a recently engineered floxed-*Gabrd* mouse (Lee and Maguire, 2013) and the PV-IRES-Cre line (JAX Stock # 008069), we specifically deleted the δ dubunits of GABA$_A$Rs from PV + INs (PV-*Gabrd*$^{-/-}$) to examine potential changes in ovarian cycle-linked modifications in γ oscillations magnitude in the absence of δ-GABA$_A$Rs on PV-INs. Our findings identify a possible underlying cause for the different degrees of cognitive impairment experienced by some women at various phases of the ovarian cycle.

MATERIALS AND METHODS

ANIMAL HANDLING

In this study we used adult (15–20 week-old) female and male C57BL/6J mice, WT (*Cre*$^{-/-}$) or mice lacking δ-GABA$_A$Rs specifically on PV-INs (PV-*Gabrd*$^{-/-}$), generated by crossing PV-*Cre* (PV-IRES-Cre line; JAX Stock # 008069) and *Gabrd*-flox mice (generous gift of Dr. Jamie Maguire, Tufts University; Lee and Maguire, 2013) both back-crossed for >10 generations on C57BL/6J background. The δ-GABA$_A$Rs ablation from PV-IN was confirmed with immunohistochemical fluorescent double labeling (Ferando and Mody, in preparation, data not shown). Mice were housed with *ad libitum* access to food and water and kept on a 12-h light/dark cycle, under the care of the UCLA Division of Laboratory Animal Medicine (DLAM). All experiments were performed during the light period and according to a protocol (ARC # 1995-045-53B) approved by the UCLA Chancellor's Animal Research Committee. Genotyping was performed by Transnetyx (Memphis, TN, USA).

SURGERY

Surgeries were performed under aseptic conditions on mice weighing 25–30 g. Under isoflurane anesthesia (2–2.5% in O_2 alone) the animal was mounted into a standard Stoelting instrument stereotaxic frame with blunt ear bars. Body temperature was maintained at 37°C using a rectal probe and a water circulated heating pad. The cranium was exposed through a small midline scalp incision. The bone was dried and three holes were drilled (0.5 mm diameter) in the cranium. With the aid of a micromanipulator, two sterilized recording electrodes (PlasticsOne, stainless steel, 125 μm diameter) were lowered into hippocampal CA1 region SP, bilaterally (at stereotaxic coordinates: anteroposterior, AP, 5.5 mm; mediolateral, ML, 1.45 mm; dorsoventral, DV, 1.2 mm from brain surface). The third hole was drilled above the cerebellum to insert the ground/reference electrode. The skull surface was covered by thin layer of cyanoacrylate based glue (Insta-Cure+, Bob Smith Industries) and then dental acrylate (Ortho-Jet, Lang Dental Manufacturing Co., Inc.) was used to attach the electrode sockets to the skull surface. Immediately after surgery, the mouse was continuously monitored until recovered, as demonstrated by their ability to maintain sternal recumbency and to exhibit purposeful movement. During the recovery period after surgery, warm saline solution (0.01–0.02 ml/g/twice/day) was administered subcutaneously to prevent dehydration. To prevent any infection around the implant we topically administered Neosporin for 7 days. For analgesia, 0.1 mg/kg of buprenorphine was injected subcutaneously prior to surgery. Buprenorphine injections were continued following the surgery every 12 to 48 h.

OVARIAN CYCLE INDUCTION AND MONITORING

Female virgin mice are generally anovulatory or have irregular cycles, unless exposed to male pheromones. In the present study ovarian cycle was induced in previously anovulatory virgin adult mice (15–20 week-old) by a single exposure to male bedding, and monitored by means of vaginal impedance measurements and vaginal smears cytological analysis, as previously described (Ramos et al., 2001; Maguire et al., 2005; Jaramillo et al., 2012;

Cushman et al., 2014). Briefly, vaginal impedance was measured daily (Estrus cycle monitor EC40, Fine Science Tools). Daily vaginal smears were fixed in methanol and stained (Giemsa Staining, Fisher Diagnostics). Diestrus and estrus were defined as 3 days prior and 1 day after vaginal impedance peak, respectively, and by vaginal cytology profile (e.g., **Figure 5A**). Mice were tested in either diestrus (high plasma progesterone) or estrus (low plasma progesterone) phase of their ovarian cycle.

IN VIVO CHRONIC SIMULTANEOUS VIDEO AND LOCAL FIELD POTENTIAL RECORDINGS

Seven to ten days after the animals had fully recovered from the surgery, chronic simultaneous video and local field potential recordings were carried out continuously (24 h a day) for 1–3 weeks. Video observation was performed through an infrared USB camera mounted on the top of the recording cage. The video was recorded using the open source iSpy software, which calculates in real time the percentage deviation between consecutive frames and generates a text file (activity data) containing time-stamped information on the percentages of frame-to-frame deviation values.

Local field potentials were recorded with a custom-made miniature dual headstage amplifier connected to the electrode sockets mounted on the animal's head and then wired to an electrical commutator (Dragonfly Inc., or Pinnacle Technology Inc.). The signals from the commutator were fed through a 16-channel extracellular amplifier (A-M Systems model 3500) with a gain of 1,000. Signals were low-pass filtered at 1,000 Hz and sampled at 2,048 s^{-1} per channel, using a National Instruments A/D board.

Continuous data acquisition was carried out using Igor NIDAQ tool (Wavemetrics, Lake Oswego, OR, USA) and data were saved into separate files every week. Activity graphs deriving from the video recordings and corresponding local field potentials were loaded into a custom made software (written in Igor64, Wavemetrics, Lake Oswego, OR, USA) that aligned the two recordings to determine sleep and wake cycles.

IMMUNOHISTOCHEMISTRY AND MICROSCOPY

Brains were processed and tissue stained as previously described (Ferando and Mody, 2013a). Briefly, mice were transcardially perfused with 4% paraformaldehyde in 0.12 M phosphate buffer, pH 7.3. Fixed brains were sectioned at –16°C with a cryostat (coronal, 35 μm). All sections used for the same analyses (e.g., **Figures 4C,D**) were processed in parallel.

For diaminobenzidine (DAB) δ-GABA$_A$Rs stain: quenching of endogenous peroxidases was done in H_2O_2 (3% in methanol, 30 min). Slices were blocked in 10% normal goat serum (NGS), 2 h, incubated with anti-δ-GABA$_A$R antibody (1:500; generous gift from Dr. Werner Sieghart, Medizinische Universität, Wien, Austria) overnight, then with biotinylated goat anti-rabbit antibody (1:200; Vector Laboratories), 4 h. Amplification was done with HRP-conjugated avidin enzyme complex (ABC Elite; Vector Laboratories), 30 min. Signal was developed with DAB (Vector Lab). All steps were done at room temperature.

Bright field microscopy: digital images were collected with an Axioskop 2 Microscope, AxioCam digital camera system and AxioVision 4.8 software (Zeiss). For the same magnification images were taken under identical conditions of brightness and exposure time. Intensity of labeling was measured as optical density (OD) of the region of interest (ROI) using NIH ImageJ software. For CA1 and CA3 INs the ROI was the soma of all visually identified INs within 30 μm of the pyramidal cell layer, for DGML, CA1 stratum oriens (SO) and radiatum (SR), areas of approximately the same size of identified INs were circled and OD was measured. Reported OD values (represented in arbitrary units, AU) are mean ± SEM (**Table 1**). Statistical significance was determined with the use of statistical tests specified in each section.

ELECTROPHYSIOLOGY DATA ANALYSIS

Video and local field potential recordings were started after 2–3 days of habituation to the recording home cage. Data were analyzed in 24 h long epochs. Local field potential recordings were filtered in the δ range (1–4 Hz) and the δ magnitude was calculated using the Hilbert transformation. Both activity values deriving from the video data and delta magnitudes were binned at 1 s bin width. The binned activity values were plotted against the binned delta magnitudes for a 24 h-long session. Based on the point clouds, 3 clusters were separated (low δ + high activity, low δ + low activity, high δ + low activity) and thresholds for δ and activity values between the clusters were determined (**Figure 1A**).

Using these thresholds a custom made software (written in Igor64, Wavemetrics, Lake Oswego, OR, USA) categorized every second of the long local field potential recording into one of the following 4 groups: movement (activity + low δ), NREM sleep (zero activity + high δ), REM sleep (zero activity + low δ) and a fourth category which consisted of segments that could not be determined (ND; **Figure 1B**).

The definition of REM sleep was further narrowed by accepting only zero activity + low δ segments longer than 20 s that were adjacent to a segment characterized by high δ + zero activity (putative NREM sleep) phase. The detected REM segments were filtered in the θ (5–12 Hz) and high γ (63–120 Hz) range and θ phases and γ magnitudes were calculated using Hilbert transforms (**Figure 2C**). θ phase coupled γ amplitudes were determined by calculating the difference between the min and max values of the θ phase triggered average γ magnitude. On **Figures 2, 3,** and **5** the γ amplitudes were normalized to the mean values across all days and then plotted as a function of days. For male and non-cycling female mice the differences in γ amplitudes were determined at 3 day intervals that approximated the time difference between the estrus and diestrus phases of cycling female mice.

Time-frequency representation of the signal (**Figure 2C**) was performed using the Morlet wavelet transform. The magnitude of the wavelet transform was plotted as a function of time and frequency, with warmer colors representing increasing magnitude.

STATISTICS

Data are expressed as mean ± SEM. For group comparisons we used one-way ANOVA with Tukey's *post hoc* test corrected for multiple comparisons. $p < 0.05$ were accepted as significant differences.

RESULTS

THE AMPLITUDE OF HIPPOCAMPAL γ OSCILLATIONS RECORDED *IN VIVO* FLUCTUATES WITH PHASES OF THE OVARIAN CYCLE

In light of current evidence about cognitive performance fluctuations over the ovarian cycle in both women and rodents, it is remarkable that γ oscillations, a network activity that has been implicated in memory and encoding (Singer, 1993; Colgin and Moser, 2010; Uhlhaas et al., 2011; Buzsáki and Wang, 2012), have not been examined in relationship to the menstrual cycle in women or to the ovarian cycle in rodents. We therefore sought to measure γ oscillations coupled to θ rhythms in cycling female WT mice. We focused on REM θ – γ episodes, because this state is relatively easy to identify, shows consistent θ phase γ amlpitude coupling and appears to be linked to emotional information processing and memory consolidation (Montgomery et al., 2008; Walker, 2009; Scheffzük et al., 2011).

To test possible ovarian cycle related changes in γ activity during REM θ-γ episodes, in the first set of experiments continuous video local field potential recordings were performed for 2–3 weeks in cycling female WT mice. REM phases were detected and the average θ phase coupled γ amplitudes were calculated for each consecutive day. Plotting the normalized γ amplitudes revealed a characteristic fluctuation, which correlated with the stage of the ovarian cycle determined by vaginal impedance or cytology (**Figure 2A**).

Comparing the distribution of γ amplitudes for a large number ($n > 10$/day) of REM segments in estrus and diestrus indicated a shift toward higher γ amplitudes during estrus (averages of normalized γ amplitudes across animals: 1.21 ± 0.04 for estrus, 0.82 ± 0.04 for diestrus, $n = 2$ mice). Comparing the wavelet spectrogram of sample REM segments of a representative estrus and a diestrus day, revealed a more prominent presence of higher γ frequencies during estrus (**Figure 2C**). The average FFT spectra of all REM phases during an estrus and diestrus day showed a clear shift toward higher γ frequencies (**Figure 2D**). To investigate this alteration in γ oscillations throughout the study we focused on the θ phase coupled γ activity (γ amplitude) in the frequency range (63–120 Hz) where the largest shifts were found in the FFT spectra.

THE AMPLITUDE OF γ OSCILLATIONS IS CONSTANT IN WT MALE AND NON-CYCLING FEMALE MICE

To ensure that the dependence of the observed γ rhythm fluctuations on the stages of the ovarian cycle was not a random phenomenon, we also investigated possible alterations in γ oscillation magnitude over several days in WT males (averages of normalized γ amplitudes across animals: 1.00 ± 0.01, 0.98 ± 0.01 for the first and last days of a shifting 3-day window, $n = 2$ mice; **Figure 3B**) and non-cycling females (averages of normalized γ amplitudes across animals: 1.02 ± 0.02, 0.98 ± 0.03 for the first and last days of a shifting 3-day window, $n = 2$ mice; **Figure 3B**). During REM, θ coupled γ amplitudes did not reveal fluctuations over similar temporal windows in these 2 groups, demonstrating that in the absence of ovarian cycle-linked hormonal fluctuations there are no periodic changes in γ activity.

THE OVARIAN CYCLE IS ASSOCIATED WITH δ-GABA$_A$R SUBUNIT EXPRESSION CHANGES IN PRINCIPAL CELLS AND INTERNEURONS OF THE HIPPOCAMPUS

We have previously shown how γ oscillations dynamics *in vitro* are controlled by δ-GABA$_A$Rs expressed on PV-INs (Mann and Mody, 2010) and plasticity of these receptors during pregnancy alters network excitability and γ oscillations frequency (Maguire et al., 2009; Ferando and Mody, 2013a). δ-GABA$_A$Rs expression modulation during the ovarian cycle has been described in hippocampal DGGCs with direct consequences on the tonic GABA conductance, anxiety and cognitive performance (Maguire et al., 2005; Maguire and Mody, 2007; Cushman et al., 2014). Specifically, δ-GABA$_A$Rs expression is decreased in the hippocampus of WT mice during estrus, when plasma progesterone levels

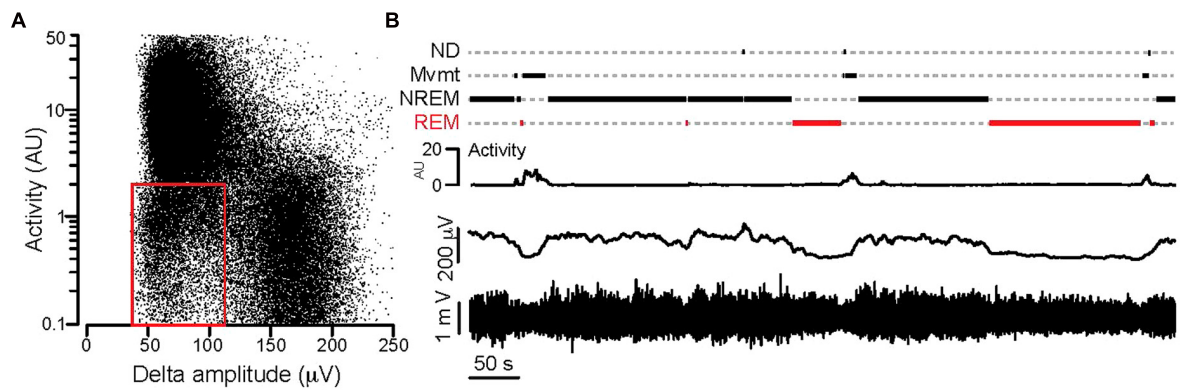

FIGURE 1 | Separation of behavioral states based on synchronous video and local field potential recordings. (A) Activity values plotted against the Hilbert magnitudes in the δ frequency range (1–4 Hz) for each 1 s long epoch (total: 86,400 epochs) during a full day of synchronous video-local field potential recording. Note the appearance of three clusters in the point cloud. The *red rectangle* delineates the point cluster corresponding to the putative REM sleep. **(B)** Separation of behavioral states based on combined activity and electrographic thresholds over an ∼12 min period. *Bottom*: local field potential recording, *above*: 1 s binned Hilbert magnitude of the δ-frequency range, *above*: activity graph, *top*: step function showing behavioral states based on activity and δ magnitude values (see text for details; REM, REM-sleep; NREM, NREM-sleep; Mvmt, movement; ND, not determined). For the detailed explanation of the applied calculations please refer to the Sections "Materials and Methods," and "Electrophysiology Data Analysis."

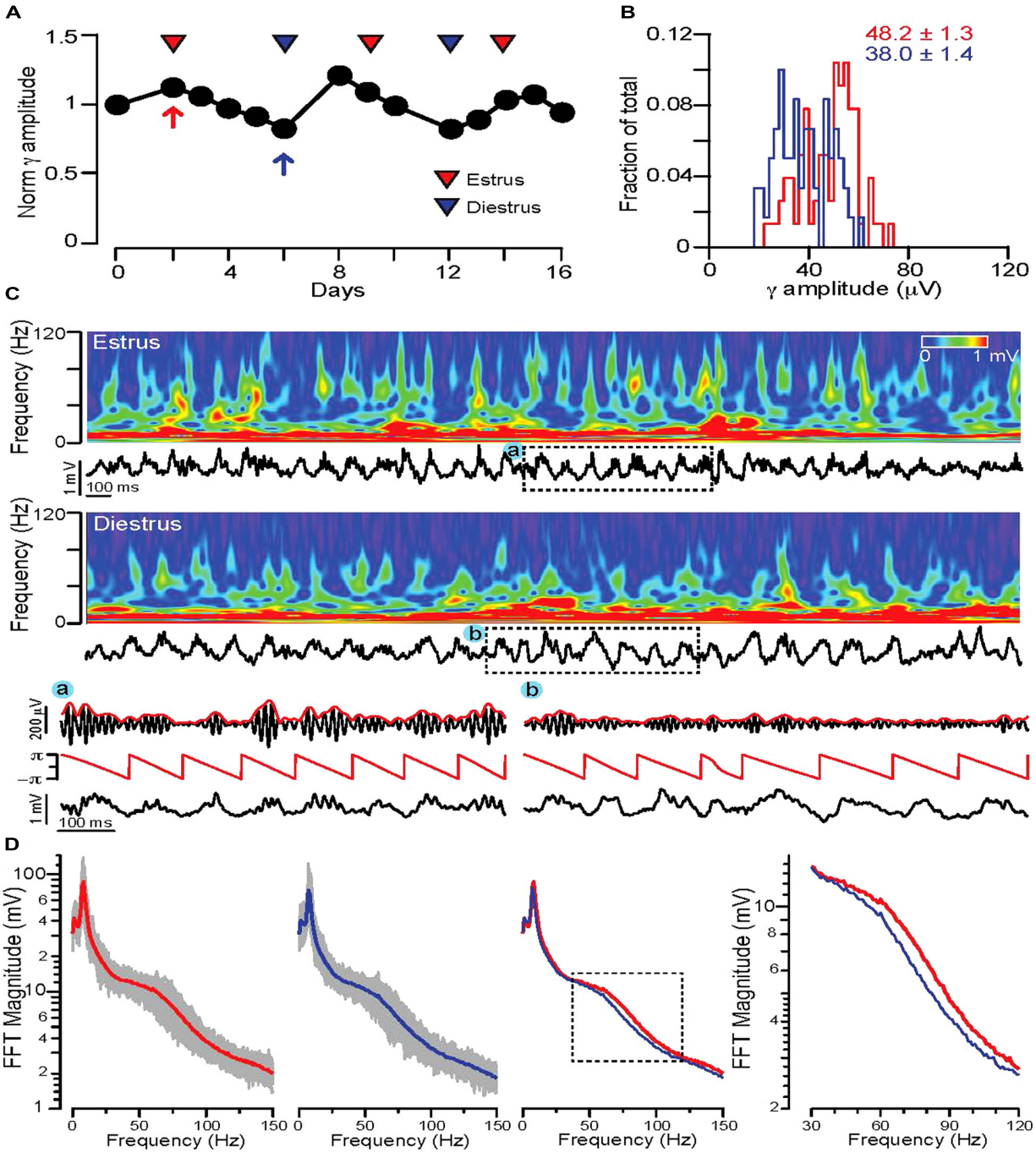

FIGURE 2 | Fluctuations in REM sleep γ oscillation magnitudes correlate with stages of the ovarian cycle in WT mice. (A) Diagram showing average normalized θ phase coupled γ amplitudes during REM sleep on consecutive days in a WT cycling female mouse. Red and blue triangles indicate estrus and diestrus days, respectively. **(B)** Distribution of γ amplitudes (binned at 2 μV) recorded in all REM phases of a single day of estrus (red) and one of diestrus (blue). The same days are indicated on **(A)** with red (estrus) and blue (diestrus) arrows. Note the shift of the distribution toward larger values during estrus. Colored numbers indicate mean ± SEM of the corresponding histograms. **(C)** Top rows: 4 s long local field potential recordings with the corresponding wavelet spectra during REM sleep of estrus (top) and diestrus (bottom). **(A)** and **(B)**: θ phase and γ magnitude components of local field potential segments indicated by dashed rectangles. *Below*: Hilbert phases of the θ (5–12 Hz) frequency range, filtered trace in the γ frequency range (63–120 Hz, black) with the corresponding Hilbert magnitudes (red). **(D)** FFT spectra of local field potential recordings of all REM phases during an estrus (leftmost diagram) and a diestrus day (second from left). Gray traces indicate the FFT spectra of individual REM phases, thick lines are the average FFT spectra on an estrus (red) or diestrus (blue) day. Third diagram from left shows the superimposed two average FFT spectra (estrus: red, diestrus: blue) for comparison. The area marked by dotted lines is enlarged on the diagram on the right to show the segments of the average FFT spectra where the largest deviation appears in γ activity. For the detailed explanation of the applied calculations please refer to the Sections "Materials and Methods," and "Electrophysiology Data Analysis."

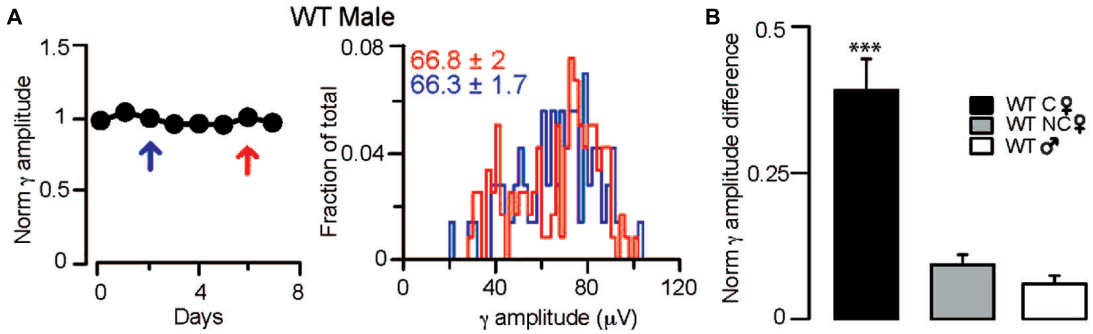

FIGURE 3 | Fluctuations in γ oscillation amplitudes recorded during REM sleep are absent in males and non-cycling WT females. (A) *Left*: diagram showing normalized γ amplitudes during REM sleep on consecutive days in a WT male mouse. *Right*: distribution of γ amplitudes (binned at 2 μV) in all REM phases recorded on two separate measurements taken 3 days apart. The 2 days are indicated on the left with red and blue arrows. Colored numbers indicate mean ± SEM of the corresponding γ amplitude histograms. **(B)** Summary data showing the absolute values of the differences between the normalized γ amplitudes during REM sleep in different groups of mice. In the cycling WT females the difference was calculated between days of diestrus and estrus. In WT males and non-cycling females the differences in γ amplitudes were determined at 3-day intervals that approximated the time difference between the estrus and diestrus of cycling female mice. Asterisks indicate significant difference from all other groups in a Tukey's multiple comparison test following a one-way ANOVA.

are low. The tonic GABA conductance recorded in DGGCs is also decreased, and mice exhibit higher degrees of anxiety and trace fear conditioning during this stage of the ovarian cycle, indicating the functional nature of the observed GABA$_A$R plasticity. In these studies, the plasticity of δ-GABA$_A$Rs over the ovarian cycle has been demonstrated in whole hippocampal western blot analyses, and by immunohistochemistry in the periaqueductal gray region (Lovick et al., 2005). In the hippocampus, δ-GABA$_A$Rs are expressed by most principal cells and some INs, and in both cell types they show high levels of plasticity during states of altered NS production (Maguire et al., 2009; Shen et al., 2010; Ferando and Mody, 2013a).

Here, in a broad manner, we addressed the hippocampal neuronal cell-type specificity of ovarian cycle-linked fluctuating expression of δ-GABA$_A$Rs. With the use of δ-GABA$_A$Rs specific antisera, we stained brains of WT female mice perfused at different stages of their ovarian cycle. The cycles were induced and determined as previously described (Maguire et al., 2005). In a separate staining we also compared δ-GABA$_A$Rs expression in CA1 and CA3 SP-INs in cycling WT females to males and non-cycling WT females. All sections were processed in parallel to allow for accurate staining intensity comparisons.

In the hippocampus δ-GABA$_A$Rs are found in the dendritic compartments of DGGCs and to a lesser extent in CA1 PCs, but not CA3 PCs. Moreover, δ-GABA$_A$Rs are expressed by different types of IN, including neurogliaform cells of the DG and lacunosum molecular and CA3, CA1 and DG PV + INs (Ferando and Mody, 2013a,b; Yu et al., 2013). INs expressing δ-GABA$_A$Rs with their somata located in the SP or within 30 μm around the SP have been shown to have over a 95% chance of being PV+ (Ferando and Mody, 2013a; Yu et al., 2013).

We found that δ-GABA$_A$Rs expression fluctuates over the ovarian cycle in DGGCs, CA1 PCs, and CA1 and CA3 SP INs (**Figures 4A–C**; **Table 1**). In particular, during times of low plasma progesterone (estrus), staining for δ-GABA$_A$Rs is significantly lower compared to times of high plasma progesterone (diestrus), which is suggestive of a downregulation of δ-GABA$_A$Rs expression during estrus. We found that δ-GABA$_A$Rs expression on CA1 and CA3 SP-INs is similar between diestrus female and male mice, while non-cycling females have a slightly increased expression selectively in CA1 SP-INs (**Figure 4D**; **Table 1**).

OVARIAN CYCLE-LINKED FLUCTUATIONS IN γ OSCILLATIONS AMPLITUDES DEPEND ON THE PRESENCE OF δ-GABA$_A$RS ON PV-INs

Since more than 95% of SP INs that express δ-GABA$_A$Rs also express PV (Ferando and Mody, 2013a), the observed plasticity in δ-GABA$_A$Rs through the ovarian cycle is likely to influence the functioning of PV-INs. Oscillations induced in brain slices in the γ frequency have been shown to be controlled by δ-GABA$_A$Rs of INs (Mann and Mody, 2010).

In order to address possible functional correlates to the observed δ-GABA$_A$Rs plasticity on CA1 and CA3 SP INs, we generated mice that lack the δ subunit of the GABA$_A$R selectively in PV + INs. These mice lose the great majority of δ-GABA$_A$Rs staining in CA1 and CA3 SP and its close proximity (within 30 μm), which confirms the previously described preferential distribution of δ-GABA$_A$Rs on PV-INs in these areas (Ferando and Mody, in preparation, data not shown). However, the mice have normal ovarian cycles as indicated by the vaginal smears of WT and PV-*Gabrd*$^{-/-}$ mice in diestrus and estrus. When induced with litter carrying the smell of male urine, PV-*Gabrd*$^{-/-}$ females exhibit regular ovarian cycling that can be ascertained with the use of both vaginal impedance measurements and cytological analysis of vaginal smears (Maguire et al., 2005). Their smears are indistinguishable from those of WT mice; i.e., the diestrus phase is characterized by small parabasal cells, large intermediate cells and abundant mucus, while estrus is characterized by large cornified anucleated superficial cells (**Figure 5A**).

Once we established that PV-*Gabrd*$^{-/-}$ females have regular ovarian cycles, we went on to measure γ oscillations coupled to θ

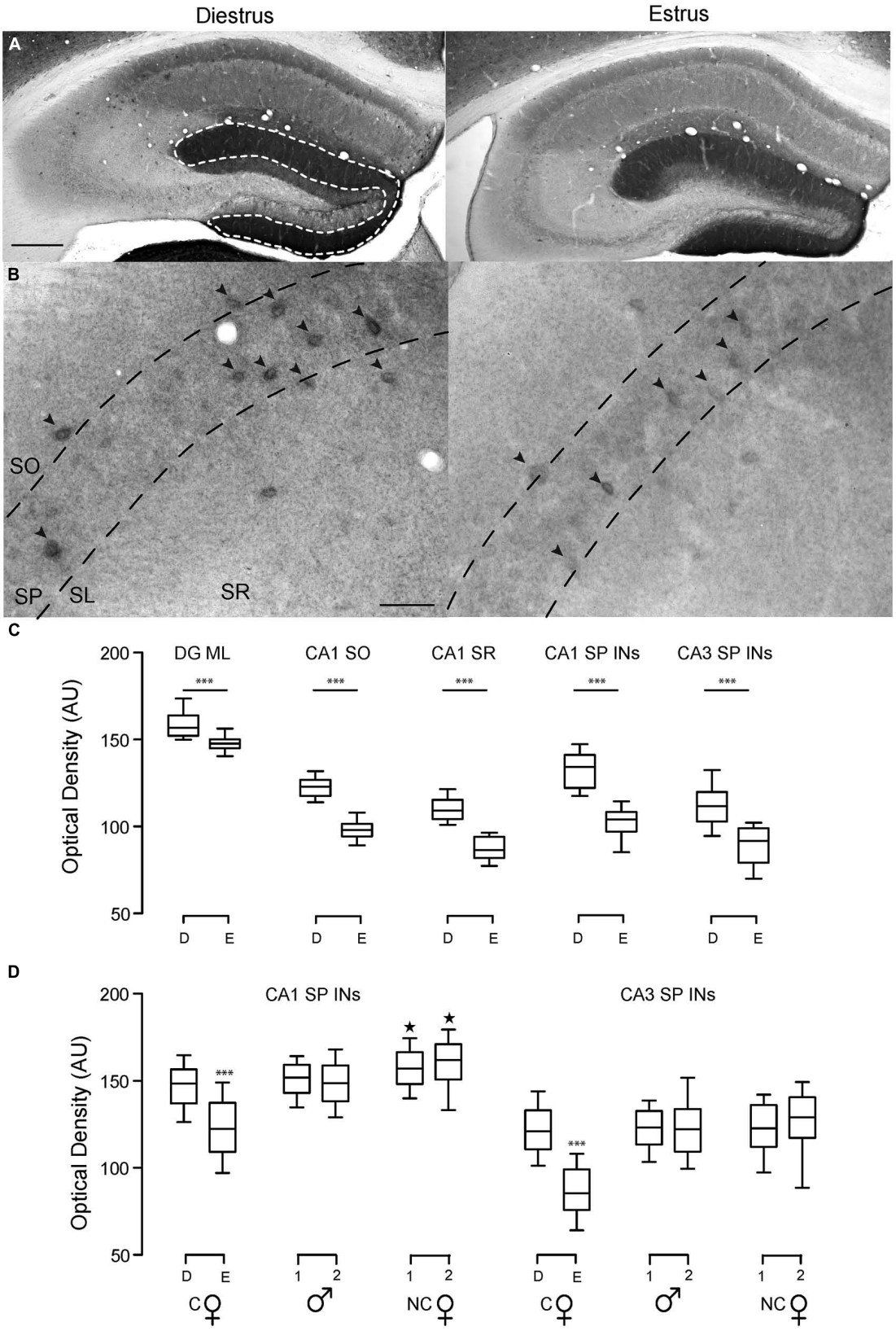

FIGURE 4 | Continued

FIGURE 4 | Continued
Hippocampal δ-GABA$_A$R plasticity at different stages of the ovarian cycle. Representative bright field images of hippocampal DAB staining showing δ-GABA$_A$R expression patterns in WT cycling females at different stages of the ovarian cycle. In the hippocampus, δ-GABA$_A$Rs are heavily expressed in the molecular layer of the dentate gyrus (DGML) and on numerous INs including neurogliaform cells and PV-INs CA1 strata oriens and radiatum (CA1 SO and SR). Expression in CA1 PC dendrites in the SR is less prominent, but noticeable. **(A)** δ-GABA$_A$R expression in DGGCs and CA1 pyramidal cells is lower in estrus compared to diestrus, and this is reflected in the staining intensity in the DGML and CA1 SO and SR. Scale bar 200 μm. **(B)** In CA1 and CA3 SP INs are strongly labeled with δ-GABA$_A$R. Optical densities of δ-GABA$_A$R expression were measured only in these INs (black arrowheads). Labeling of INs is weaker during estrus, which suggests lower δ-GABA$_A$Rs expression on SP-INs (95% of which are PV-INs) during this stage of the ovarian cycle. Scale bar 50 μm. **(C)** Optical density measurements are in AU. Box plots represent the 25th and 75th percentile, the line in the middle is the median, and the 10th and 90th percentile are indicated by the error bars. ***$p < 0.0001$ difference from all other groups. **(D)** Optical density measurements of CA1 and CA3 SP INs in slices of cycling WT female mice (C♀) in diestrus (D) or estrus (E), male mice (♂) and non-cycling WT female mice (NC♀). δ-GABA$_A$Rs expression on SP-INs during estrus is lower than any other group in both CA1 and CA3 (***$p < 0.0001$); in the CA1 the two NC♀ groups are both higher than any other group in CA1 (*$p < 0.0001$). Significance levels were established by one-way ANOVA followed by Tukey's multiple comparisons test. All sections for the separate measurements in **(C)** and **(D)** were processed together to allow for densitometric comparisons.

rhythms during REM sleep periods during the estrus and diestrus stages of the ovarian cycle, as we have done for WT females. Interestingly, we could not observe any fluctuations in γ oscillation magnitude in cycling PV-*Gabrd*$^{-/-}$ female mice (averages of normalized γ amplitudes across animals: 1.02 ± 0.04 for estrus, 0.97 ± 0.03 for diestrus, $n = 2$ mice) indicating the requirement of intact δ-GABA$_A$Rs on PV + INs for the observed fluctuations in γ amplitudes. Comparing the difference in γ amplitudes calculated between diestrus and estrus or between measurements taken 3 days apart (for explanation, see Materials and Methods) in the 4 groups revealed significant fluctuation in the γ amplitudes in cycling WT mice (based on 6 estrus – diestrus days for cycling WT and 4 estrus – diestrus days for cycling PV-*Gabrd*$^{-/-}$ mice, 2 animals in each group $F_{(3,21)} = 3.072$, $p < 0.0001$).

DISCUSSION

In this study we describe fluctuations of γ oscillation amplitudes recorded during REM sleep *in vivo* that are coupled to distinct phases of the ovarian cycle. Such periodic fluctuations in γ oscillation amplitudes were not present in male or non-cycling female mice. The γ amplitude fluctuations are inversely related to the level of expression of δ-GABA$_A$Rs hippocampal INs located in the SP, and critically depend on the presence of δ-GABA$_A$Rs on PV-INs. Although the broad shape of oscillation frequency spectra recorded *in vivo* makes it difficult to detect a precise shift in a single coherent frequency peak, we nevertheless noted a shift to the right of the recorded spectra during estrus in WT females, so that higher frequencies became more powerful. This finding is consistent with previous *in vitro* studies describing higher γ oscillations frequency during periods of low δ-GABA$_A$Rs expression on PV-INs (Mann and Mody, 2010; Ferando and Mody, 2013a). Our findings are also consistent with our previous *in vivo* results showing increased γ oscillatory power in the olfactory bulb, after selective ablation of GABA$_A$Rs on INs (Nusser et al., 2001).

As NS fluctuate over the cycle, so does the expression of the highly NS sensitive δ-GABA$_A$Rs in different neurons of the hippocampus. Interestingly, levels of δ-GABA$_A$Rs in hippocampal SP-INs at diestrus are comparable to those found in male mice, whereas δ-GABA$_A$Rs expression decreases during estrus. Non-cycling females appear to have slightly higher δ-GABA$_A$Rs levels than males, selectively on CA1 SP INs. Specific genetic and optogenetic manipulations of PV-INs have cemented their role in the local generation of γ oscillations (Sohal et al., 2009; Wulff et al., 2009; Korotkova et al., 2010; Carlén et al., 2011; Zheng et al., 2011; Lasztoczi and Klausberger, 2014). In line with these findings, our observations merely point out that hippocampal γ oscillation magnitude also depends on the expression of δ-GABA$_A$Rs on these neurons. These receptors are extremely plastic, dramatically changing their expression levels within a few days during

Table 1 | Details of δ-GABA$_A$Rs expression levels in different cell types of the hippocampus over the ovarian cycle, detected by immunohistochemistry.

ROI or INs	Status	OD (Mean ± SEM; AU)	ROI or INs (*n*)	Sections (*n*)	Mice (*n*)
DGML	Diestrus	157 ± 2	20	6	2
	Estrus	147.7 ± 1.1*	20	6	2
CA1 SO	Diestrus	122.4 ± 1.4	20	6	2
	Estrus	97.8 ± 1.3*	20	6	2
CA1 SR	Diestrus	109 ± 1.6	20	6	2
	Estrus	86.9 ± 1.5*	20	6	2
CA1 SP INs	Diestrus	132.6 ± 1.4	67	6	2
	Estrus	102 ± 1.4*	64	6	2
CA3 SP INs	Diestrus	112.7 ± 2.1	44	6	2
	Estrus	88 ± 1.7*	55	6	2
CA1 SP INs	Diestrus	145.5 ± 1.4	100	8	2
	Estrus	121.5 ± 1.8*	104	8	2
	Males1	149.5 ± 1.2	107	8	2
	Males2	147.6 ± 1.4	109	8	2
	NC females1	155.8 ± 1.2*	114	8	2
	NC females2	177.7 ± 1.6*	113	8	2
CA3 SP INs	Diestrus	119.6 ± 2	63	8	2
	Estrus	84.4 ± 2*	64	8	2
	Males1	120 ± 1.9	63	8	2
	Males2	120.2 ± 2.1	74	8	2
	NC females1	119.7 ± 2.1	59	8	2
	NC females2	124.1 ± 2.5	66	8	2

Summary by hippocampal region of interest (ROI) or INs of optical density mean values in arbitrary units (AU) ± SEM, and n's for diestrus and estrus in WT cycling females, WT non-cycling females and males. Asterisks denote significance (see **Figure 4** *for statistical tests).*

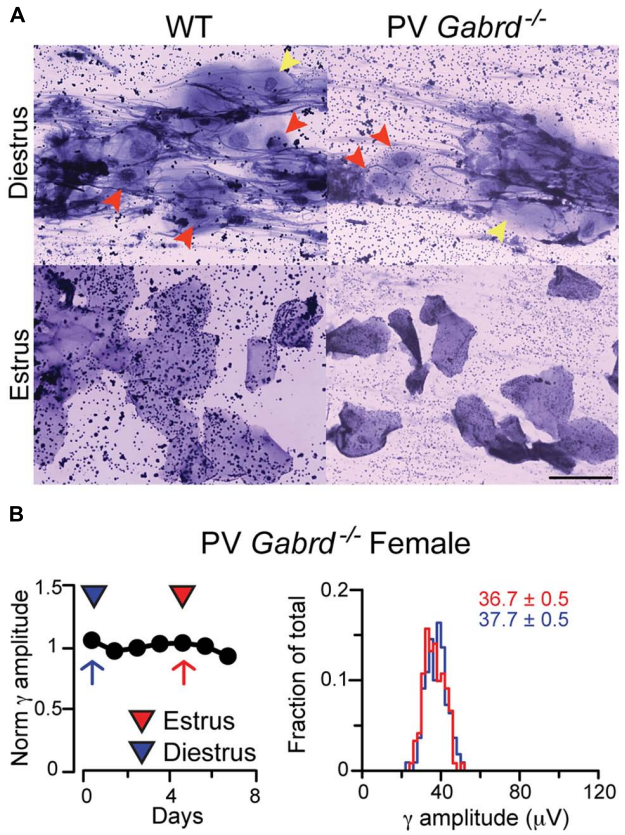

FIGURE 5 | Female PV-*Gabrd*$^{-/-}$ mice cycle regularly but lack ovarian cycle-dependent modulation of REM phase γ oscillation amplitudes. **(A)** Ovarian cycle stage was determined by vaginal impedance measurements and cytological analysis of vaginal smears. PV-*Gabrd*$^{-/-}$ mice had a similar cytological panel to WT mice at different stages. Diestrus was determined by the presence in the smear of abundant mucus, small parabasal cells (red arrowheads) and large intermediate cells (yellow arrowheads). Both cell types are absent in estrus, when smears are typically characterized by large polygonal superficial cells, mostly fully cornified. Scale bar 5 μm. **(B)** Left: diagram showing normalized γ amplitudes during REM sleep on consecutive days in a regularly cycling PV-*Gabrd*$^{-/-}$ female. Red and blue triangles indicate estrus and diestrus, respectively. *Right*: distribution of γ amplitudes (binned at 2 μV) recorded during all REM sleep episodes for an entire day of estrus (red) and one of diestrus (blue). The 2 days are marked on the left with red (estrus) and blue (diestrus) arrows. Colored numbers indicate mean ± SEM of the corresponding histograms. Note the narrow variance of the γ amplitude distributions during both phases of the ovarian cycle.

hormonal alterations of the ovarian cycle. The precise consequences of fluctuating γ oscillations on memory and cognitive performances may not always be easily predictable, although reports suggest enhanced memory performance to be correlated with higher γ frequency band magnitude (Lu et al., 2011).

A prediction based on our studies would be that cognitive processes in females would be enhanced during the low progesterone phase of the ovarian cycle (estrus) when γ oscillations are increased. Indeed, several studies reported higher hippocampus-mediated learning and memory performance and increased anxiety levels in female rodents during the estrus phase of the ovarian cycle, whereas animals in diestrus performed similar to males (Maguire et al., 2005; Walf et al., 2006; Moore et al., 2010; van Goethem et al., 2012; Cushman et al., 2014). Although at present it is unknown whether similar alterations in PV-IN δ-GABA$_A$Rs also take place in humans, menstrual cycle-dependent variations in memory performance are not uncommon (Bayer et al., 2014). It also remains to be determined whether a higher cognitive capacity during the preovulatory phase may provide any evolutionary advantage.

In addition to INs, δ-GABA$_A$Rs are also expressed on most principal cells of the hippocampus (Sperk et al., 1997; Glykys et al., 2007; Milenkovic et al., 2013; Ferando and Mody, 2013a). Although δ-GABA$_A$Rs expression on CA1 PCs is modulated across the ovarian cycle (**Figure 4C**), this does not seem to result in appreciable changes in network level activity, as also supported by previous studies reporting comparable CA1 PC tonic conductances in diestrus and estrus (Maguire et al., 2005). This is not surprising as in these cells 70% of the total tonic inhibition is mediated by α5-GABA$_A$Rs, which have been shown to easily compensate for δ-GABA$_A$Rs loss (Glykys et al., 2008). In contrast, the tonic GABA conductance of hippocampal INs seems to be solely mediated by δ-GABA$_A$Rs (Glykys et al., 2008). It is interesting to note the narrow variance of γ oscillation amplitudes in PV-*Gabrd*$^{-/-}$ mice. This phenomenon will need further investigation, as it is possible that complete lack or insufficient levels of δ-GABA$_A$Rs on PV-INs may allow for restricted degrees of modulation of the γ oscillatory amplitudes, resulting in potentially disruptive effects on cognitive function. Unfortunately, our study using simple single site recordings does not permit accurate comparisons of the γ oscillation amplitudes across animals. Future multi-site recordings and current source density analyses will be required to ascertain any potential regional differences in the magnitude of γ oscillations between WT and PV-*Gabrd*$^{-/-}$ animals.

The molecular mechanisms responsible for δ-GABA$_A$Rs plasticity during the ovarian cycle, or following steroid fluctuations in general, are unknown but may involve protein phosphorylation, and transcriptional modifications (Choi et al., 2008; Jacob et al., 2008; Abramian et al., 2010; Saliba et al., 2012). Recently, intracellular Cl^- itself has been proposed to function as a messenger for plasticity of different GABA$_A$Rs subunits (Succol et al., 2012). Nonetheless, NS synthesis is a necessary event for δ-GABA$_A$Rs modulation over the ovarian cycle (Maguire and Mody, 2007).

The lack of γ oscillation modulation in PV-*Gabrd*$^{-/-}$ *in vivo* suggests that pathological alteration in the normal phsyiology of IN-δ-GABA$_A$Rs through the ovarian cycle may have important consequences on how information is processed at the network level, and may predispose to pathological conditions if combined with aggravating events that lead to altered NS production or inadequate IN δ-GABA$_A$Rs plasticity. Therefore, the development of δ-GABA$_A$Rs specific drugs to selectively control IN function may be a novel future approach to the treatment of these symptoms in women with premenstrual syndrome (PMS) and PMDD.

AUTHOR CONTRIBUTIONS

Albert M. I. Barth, Isabella Ferando, and Istvan Mody designed research; Albert M. I. Barth performed electrophysiology experiments; Isabella Ferando performed immunohistochemistry

experiments and determined the ovarian cycle stages; Albert M. I. Barth, Isabella Ferando, and Istvan Mody analyzed data; Isabella Ferando and Istvan Mody wrote the paper.

ACKNOWLEDGMENTS

This research was supported by an American Heart Association Postdoctoral Fellowship to Albert M. I. Barth, by NIH grants MH076994, NS030549, and the Coelho Endowment to Istvan Mody We are indebted to Jamie Maguire, Ph.D. (Tufts University) for kindly providing the floxed-*Gabrd* mice. We would like to thank R. Main Lazaro for expert technical assistance and to members of the ModyLab for helpful comments during data presentation sessions.

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

Received: 16 April 2014; accepted: 21 July 2014; published online: 11 August 2014.
*Citation: Barth AMI, Ferando I and Mody I (2014) Ovarian cycle-linked plasticity of δ-GABA$_A$ receptor subunits in hippocampal interneurons affects γ oscillations in vivo. Front. Cell. Neurosci. **8**:222. doi: 10.3389/fncel.2014.00222*
This article was submitted to the journal Frontiers in Cellular Neuroscience.

frontiers in
CELLULAR NEUROSCIENCE

REVIEW ARTICLE
published: 20 June 2014
doi: 10.3389/fncel.2014.00166

Regulation of adult neurogenesis by GABAergic transmission: signaling beyond $GABA_A$-receptors

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In the adult mammalian brain, neurogenesis occurs in the olfactory bulb (OB) and in the dentate gyrus (DG) of the hippocampus. Several studies have shown that multiple stages of neurogenesis are regulated by GABAergic transmission with precise spatio-temporal selectivity, and involving mechanisms common to both systems or specific only to one. In the subgranular zone (SGZ) of the DG, GABA neurotransmitter, released by a specific population of interneurons, regulates stem cell quiescence and neuronal cell fate decisions. Similarly, in the subventricular zone (SVZ), OB neuroblast production is modulated by ambient GABA. Ambient GABA, acting on extrasynaptic $GABA_A$ receptors ($GABA_AR$), is also crucial for proper adult-born granule cell (GC) maturation and synaptic integration in the OB as well as in the DG. Throughout adult-born neuron development, various GABA receptors and receptor subunits play specific roles. Previous work has demonstrated that adult-born GCs in both the OB and the DG show a time window of increased plasticity in which adult-born cells are more prone to modification by external stimuli. One mechanism that controls this "critical period" is GABAergic modulation. Indeed, depleting the main phasic GABAergic inputs in adult-born neurons results in dramatic effects, such as reduction of spine density and dendritic branching in adult-born OB GCs. In this review, we systematically compare the role of GABAergic transmission in the regulation of adult neurogenesis between the OB and the hippocampus, focusing on the role of GABA in modulating plasticity and critical periods of adult-born neuron development. Finally, we discuss signaling pathways that might mediate some of the deficits observed upon targeted deletion of postsynaptic $GABA_A$Rs in adult-born neurons.

Keywords: adult neurogenesis, olfactory bulb, dentate gyrus, $GABA_A$ receptor, plasticity

ADULT NEUROGENESIS AND GABAergic SIGNALING

Brain development depends on the coordination of numerous processes that go from cell proliferation to circuit refinement. In mature brain circuits, γ-aminobutyric acid (GABA) acts as the main inhibitory neurotransmitter. It is now well known that GABA plays more than a classical inhibitory role and can function as an important developmental signal early in life. Its actions influence processes such as proliferation of neuroblasts and migration, synapse formation, and synapse plasticity. Therefore, GABAergic transmission is essential for proper brain formation and functioning. Imbalance between excitation and inhibition (E/I) due to impaired GABAergic signaling has been implicated in several diseases, such as schizophrenia, epilepsy, autism-spectrum disorders, and intellectual disability. Similarly, GABA exerts a fundamental role in regulating adult neurogenesis, which allows its effects on developing neurons to be studied in adult tissue. The role of GABAergic signaling has been long studied (Bovetti et al., 2011; Berg et al., 2013). Here we will focus on the role of $GABA_AR$ subunits plays in adult neurogenesis.

In the first part of this review, we will briefly describe the crucial phases that lead a neural stem cell to differentiate and become an adult-born neuron in the dentate gyrus (DG) of the hippocampus and in the olfactory bulb (OB). Then, we will describe the molecular organization of $GABA_A$ receptors ($GABA_AR$). Finally, we will illustrate the role of GABAergic signals regulating adult neurogenesis. Our goal is to discuss how the spatio-temporal regulation of GABAergic transmission through distinct $GABA_AR$ subtypes is involved in modulating adult neurogenesis in the OB and DG. In doing so, we will compare the two systems in order to identify common and unique mechanisms mediated by GABAergic transmission.

NEUROGENESIS IN THE ADULT BRAIN

Adult neurogenesis is the life-long continuous production and functional integration of newborn neurons in the CNS. It represents a process by which the brain can modify itself to face and adapt to external stimuli, as well as to learn and remember. In the rodent brain, adult neurogenesis is restricted to two specific neurogenic niches, the subgranular zone (SGZ) of the DG and the subventricular zone (SVZ) of the lateral ventricles (Gage, 2000; Alvarez-Buylla et al., 2001; Rakic, 2002). The steps of adult neurogenesis include proliferation of stem and progenitor cells, neuroblast fate specification and migration, neuronal differentiation, survival, and integration into the existing circuitry. These

steps are under precise spatial and temporal control, but can be modulated by both internal and external stimuli.

In the SVZ three different types of neural precursor cells have been identified: type B radial glia-like progenitors, type C transient amplifying cells and type A migrating neuroblasts. Type B cells divide slowly and give rise to transient amplifying cells and oligodendrocytes. Type C cells divide rapidly and give rise to migrating neuroblasts (Abrous et al., 2005). Neuroblasts exhibit an elongated cell body and have two radially opposed processes (Lois and Alvarez-Buylla, 1993). Before differentiating into olfactory GCs and periglomerular cells (PGCs), these neuroblasts have to migrate for a long distance through the rostral migratory stream (RMS) toward the OB (**Figure 1B**). During migration, they form a chain consisting of a group of 30–40 cells. After about 5 days, neuroblasts reach the OB, detach from the chain in the RMS and start to migrate to reach either the granule cell layer (GCL) or the glomerular layer (GL) (Luskin, 1993; Lois and Alvarez-Buylla, 1994; Lois et al., 1996). Here they form distinct populations of interneurons, mainly located in the GCL (50–75%) and to a lesser extent in the GL (25%) (Luskin, 1993). Their integration into the preexisting circuitry occurs rapidly. One or two days after entry in the GCL, OB-GCs first receive axo-dendritic inputs from local interneurons (short axon cells), mitral cells, tufted cells, and centrifugal fibers. Finally, upon entering the external plexiform layer (EPL), the dendrite starts branching and forms dendro-dendritic reciprocal contacts with mitral and tufted cells (Whitman and Greer, 2007; Panzanelli et al., 2009). PGCs rapidly migrate toward the GL, send their axon into one or several glomeruli and receive inputs from the olfactory sensory nerve and dendro-dendritic contacts from mitral and tufted cells (Shao et al., 2009). While OB-GCs are strictly GABAergic, PGC can express various transmitter phenotypes including GABAergic, dopaminergic and glutamatergic, depending on their site of origin in the SGZ/RMS (**Figure 1C**).

The SGZ of the DG contains radial glia-like quiescent neural stem cells (Type I cells), that undergo symmetric division, or give rise to intermediate progenitor cells (Type II), astrocytes or oligodendrocytes. Postmitotic intermediate progenitors differentiate as neuroblasts, which migrate a short distance into the inner GCL and differentiate as GCs. Dentate GCs receive their main excitatory input from the enthorinal cortex and provide glutamatergic inputs to hippocampal pyramidal neurons and CA3 inhibitory interneurons (**Figure 1A**).

MOLECULAR ORGANIZATION OF $GABA_A$ RECEPTORS

Among the numerous factors regulating adult neurogenesis, GABAergic signaling, primarily through $GABA_A$Rs, plays a major role. $GABA_A$Rs are ligand-gated Cl^- channels mediating most of the fast inhibitory action of GABA. $GABA_A$Rs are also permeable to HCO^{3-} (Kaila et al., 1992), which decrease the effect of inhibition of the Cl^- entry, leading to depolarization. $GABA_A$Rs are encoded by a large family of subunit genes, grouped in seven classes according to their sequence homology: α (1–6), β (1–3), γ (1–3), δ, ε, θ, π and ρ (1–3) (Macdonald and Olsen, 1994; Sieghart et al., 1999; Sieghart and Sperk, 2002). Differential assembly of these subunits in pentameric channels results in multiple $GABA_A$R subtypes with unique functional and pharmacological properties [for α1 subunit, (Kralic et al., 2006), for α3 (Studler et al., 2005), for α5 (Fritschy et al., 1997)].

$GABA_A$R subtypes mediating synaptic GABAergic transmission in mature neurons are composed of two α1, α2, α3 subunits together with two β2 or β3 and one γ2 subunit (Jacob et al., 2008). In contrast, the receptors composed of α4, α5, or α6 subunit variants, along with β subunits and δ or γ2 (located at the extrasynaptic sites) mediate tonic GABAergic transmission through ambient GABA (Kilb et al., 2013).

Receptor properties, such as trafficking or clustering, can be regulated by interactions with scaffold proteins and major signaling complexes. Gephyrin, a phospho-protein, is the main postsynaptic scaffolding protein both for GABAergic and glycinergic synapses. It is essential for stabilization of $GABA_A$Rs but also interacts with other postsynaptic proteins, like neuroligins and collybistin (Saiepour et al., 2010; Fritschy et al., 2012). It has been shown that the absence of gephyrin results in the loss of postsynaptic $GABA_A$Rs (Essrich et al., 1998; Kneussel et al., 1999). In contrast, knockout mice lacking the α1, α2 or γ2 subunits exhibit loss of gephyrin clusters (Essrich et al., 1998; Kralic et al., 2006; Patrizi et al., 2008; Panzanelli et al., 2011). *In vitro* data demonstrate that the phosphorylation state of gephyrin affects GABAergic synaptic function by regulating cluster size and density (Tyagarajan et al., 2011, 2013). Thus, abolishing the phosphorylation of residue S270 favors the formation of supernumerary synapses in cultured hippocampal neurons (Tyagarajan et al., 2011). However, it is also reported by Levi et al. (2004) that gephyrin is not strictly required for $GABA_A$R assembly, suggesting the possibility of a gephyrin- independent mechanism of inhibitory synapse development.

Collybistin, another protein which might influence the dynamic and plasticity of $GABA_A$Rs at the surface, was shown to bind gephyrin and Cdc-42, potentially affecting the remodeling of the actin cytoskeleton. Further, collybistin can bind directly to neuroligin 2, suggesting that it plays a role in maintenance of $GABA_A$R at the plasma membrane (Poulopoulos et al., 2009; Fritschy et al., 2012). These data, along with single-particle tracking studies show that the presence of synaptic and extrasynaptic $GABA_A$Rs on the plasma membrane is highly dynamic and regulated by direct or indirect interactions with postsynaptic scaffolding proteins. This feature to adapt GABAergic transmission to the differentiation of their dendrites and incoming synaptic inputs might be particularly important for developing neurons.

So far, most of the studies describing the role of different $GABA_A$R subunits are mostly based on KO mice. Despite the considerable insight into $GABA_A$R function gained from the use of KO mice, these model systems nevertheless have certain drawbacks. KO mice for $GABA_A$R subunits show compensatory effects that impact the neural circuitry, e.g., increased expression of other subunits (Kralic et al., 2006). Adult neurogenesis is moreover a process that involves maturation, integration of single cell into circuits. Given these constrains, it is important to manipulate adult generated cells independently. Recent developments have met this need with new strategies for labeling and manipulating single cells without affecting the entire circuitry. Those new strategies make use of wild type (WT) or transgenic mice model in which the injection of viral vectors in specific brain

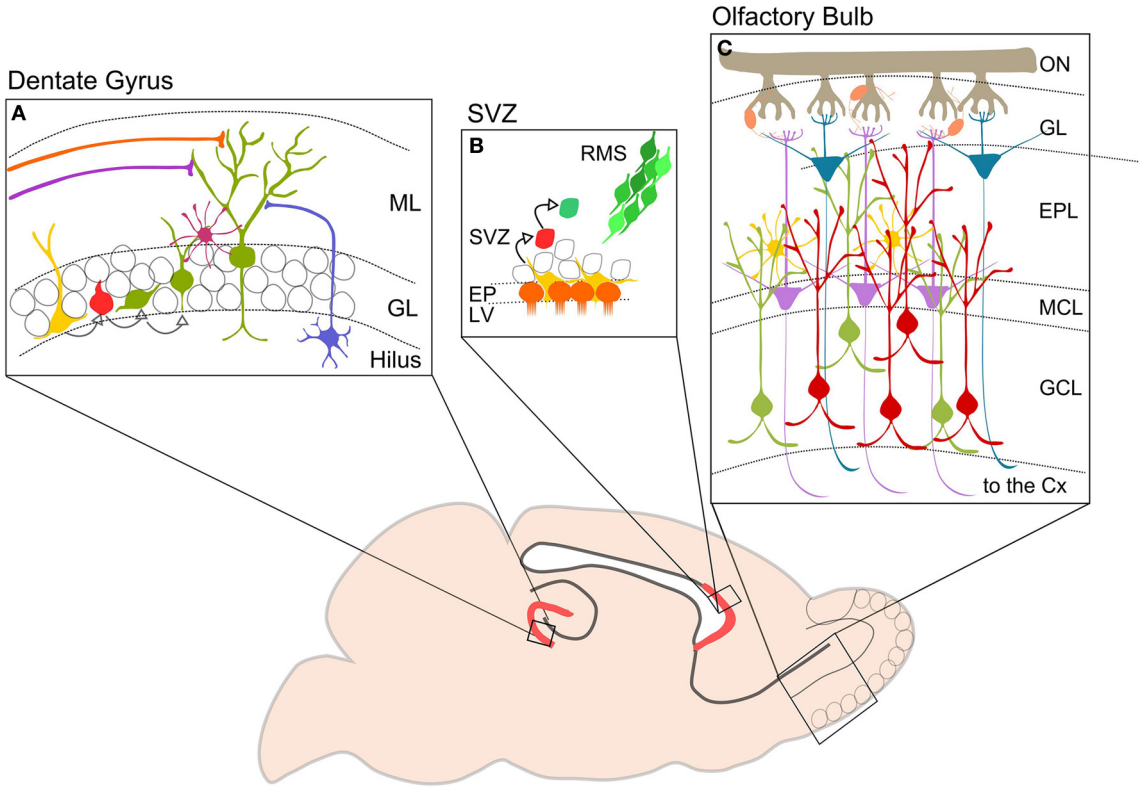

FIGURE 1 | Neurogenesis in the adult mouse brain. The picture shows in red the two neurogenerative niches: **(A)** DG of the hippocampus. Type I cells (yellow) divide to generate type II cells (red) that differentiate in immature neurons (green). DG-GCs receive GABAergic inputs from hilar interneurons (blue) and basket cells (purple), and glutamatergic inputs from lateral and medial perforant pathways (orange and violet). **(B)** SVZ. Neurogenic niche contains progenitor or type B cells (yellow) close to the ependymal cells (orange). Type b cells dived slowly and give birth to type C cells (red) which divide again in neuroblasts or type A (green). Neuroblasts migrate in chains along the RMS (from Wang and Kriegstein, 2009 modified). **(C)** Schematic representation showing OB cell types and circuitry. Axons of OSNs are collected in the ON and reach the GL where they make synapses on OB projection neurons: MCs (violet) and TCs (blue). Projection cells are modulated by interneurons, PGCs (orange) located in the GL, and perinatal and adult GCs (green and red) located in the GCL. GCs are also regulated by EPL interneurons (yellow). Axons of MCs and TCs project to higher olfactory cortical centers. ML, molecular layer; GL, granule layer; SVZ, subventricular zone; RMS, rostral migratory stream; EP, ependymal cell layer; LV, lateral ventricle; ON, olfactory nerve; ONS, olfactory sensory neuron; GL, glomerular layer; EPL, external plexiform layer; MCL, mitral cell layer; GCL, granular cell layer; CX, cortex.

areas allows labeling or manipulating of specific cell population. This can be done using different promoters or, in case of adeno-associated viruses AAV, different serotype. Because of their spatial and temporal specificity, these manipulations can be done without affecting the development of the brain. This approach is particularly useful to study adult neurogenesis, and therefore is widely used in the field.

In the adult OB and DG, distinct $GABA_AR$ subtypes are expressed in various cells types to mediate both phasic and tonic inhibition, with possible functional and pharmacological specificity among distinct circuits. The subunit repertoire of precursor cells and neuroblasts is much less well established. Here, we briefly summarize what is known about their organization in both systems (**Table 1**).

In the OB, GABAergic GCs express $GABA_AR$ contain the α2 subunit, and to a lesser extent α3 subunit (Panzanelli et al., 2009), which are responsible for mediating synaptic inhibition. Immunohistochemistry also revealed the presence of extrasynaptic $GABA_ARs$ containing the α5 subunit, along with α4 and δ subunits (Panzanelli et al., 2007). GABAergic signaling in PGCs is mainly mediated through synaptic $GABA_AR$ containing the α2 subunit and extrasynaptically through the α5-$GABA_ARs$ (Panzanelli et al., 2007).

Stewart et al. have shown by RT-PCR that precursor cells and neuroblasts in the SVZ express different $GABA_ARs$ containing the α2, α3, α4, α5; β1–3 and γ2 subunits and are activated by ambient GABA release (Stewart et al., 2002). Similarly, neurospheres from striatal neuronal progenitors express α2, α4, α5; β1–3 and γ1, γ2 1νδ γ3 subunits mRNA (Nguyen et al., 2003). Therefore, while adult-born OB-GCs are regulated by both, synaptic and extrasynaptic $GABA_ARs$ differing in subunit composition, these subunits are already expressed in OB-GC precursors.

In situ hybridization (ISH) and immunohistochemical (IHC) studies have analyzed the GABAergic subunit composition. They have found that in the DG the α2- and α4-$GABA_AR$ subunits are strongly expressed, while α1 and α5 subunits are moderate expressed in the DG (Heldt and Ressler, 2007; Hortnagl et al., 2013). The $GABA_AR$ containing the α2 or α1 subunit are responsible for phasic inhibitory transmission of GCs. At extrasynaptic sites, GCs express $GABA_ARs$ with the specific subunit

Table 1 | Distribution of various $GABA_AR$ subunits in different cell types in olfactory bulb, subventricular zone (SVZ) and dentate gyrus of the hippocampus.

	Cell type	GABA R subunit	Method	References
Olfactory Bulb	Precursor cells (SVZ)	$GABA_AR$: α2, α3, α4, α5, β1-3 αvδγ 2	RT-PCR in cultured SVZ precursor cells	Stewart et al., 2002
		$GABA_AR$: α2, α4, α5; β1-3 and γ1, γ2 1vδ γ3	RT-PCR from neurosphere	Nguyen et al., 2003
	Granule cells	$GABA_AR$: α2, α3, α4, α5, δ subunits	Immunohistochemistry	Panzanelli et al., 2007, 2009
	Periglomerular cells	$GABA_AR$: α2, α5 subunits	Immunohistochemistry	Panzanelli et al., 2007
Dentate gyrus	Type B	$GABA_AR$: α5, β3, γ2	Pharmacology	Song et al., 2012a
	Dentate gyrus:	$GABA_AR$: α1, α2, α4, α5, δ	*In situ* hybridization	Heldt and Ressler, 2007; Hortnagl et al., 2013
	Granule cells	α2, δ	Immunogold labeling in EM	Wei et al., 2003

combinations α4β2δ and α5β3γ2 (Glykys et al., 2008). In the SGZ neural stem cells respond tonically to GABA via the α5β3γ2 $GABA_AR$ composition to control their quiescent condition (Song et al., 2012a). Further it has been shown that $GABA_ARs$ containing the α4 subunit are expressed in type I cells to control their proliferation rate (Duveau et al., 2011). In conclusion, a huge diversity of $GABA_ARs$ is present in the OB and DG, although it is still not clear how the signaling through the different $GABA_AR$ subtypes is processed. We will address the relevance of distinct $GABA_AR$ subtypes in modulating critical stages of adult neurogenesis.

Most of the studies described here are performed using RT-PCR or pharmacological approaches combined to KO mice. For most of the $GABA_AR$ subunits a clear evidence of pattern expression in precursors and neuroblasts is missing. To better understand the role of the different $GABA_AR$ subunits, a deeper investigation using immunohistochemistry using specific antibodies against the different $GABA_AR$ subunits and cell markers, would be useful.

GABA SIGNALING IN ADULT NEUROGENESIS

Adult neurogenesis in the OB and in the DG differs in many aspects. Neuroblasts proliferate in two different niches; they migrate along different routes and for different distances. Then, when neuroblasts incorporate in the preexisting circuitry, they integrate with a very different timing. The functional role of adult neurogenesis, although it is still not entirely clear, is different in the two systems. Nevertheless, GABAergic neurotransmission regulates the entire process of adult neurogenesis in both systems, suggesting common mechanisms, as well as possible differences, including certain functional specificities. In the following paragraph, we aim to compare the role of GABA in regulating adult neurogenesis in the OB and DG, focusing in particular on the contribution of major $GABA_AR$ subtypes to this process (See **Table 2**).

PROLIFERATION: GABA IS A STOP SIGNAL IN CELL PROLIFERATION

In the SVZ, precursors and neuroblasts are already sensitive to neurotransmitters (Berg et al., 2013). Neonatal SVZ progenitor cells show chloride currents activated by GABA and muscimol, but they are insensitive to ATP, kainate, NMDA, and ACh (Stewart et al., 2002). The absence of synapses at the EM level suggests that GABA is synthesized and released in a non-synaptic manner by neuroblasts. This conclusion is also supported by electrophysiological and IHC analyses (Doetsch et al., 1997). Moreover, electrophysiological studies have shown that tonic release of GABA activates $GABA_ARs$ expressed by neuroblasts and stem cells (Stewart et al., 2002; Wang et al., 2003; Bolteus and Bordey, 2004; Liu et al., 2005). A second source of GABAergic innervation comes from medium spiny or aspiny neurons from the striatum. The activation of striatal neurons increases calcium level into SVZ neuroblasts (Young et al., 2014). Moreover, acute treatment with muscimol decreases BrDU incorporation in type B and C cells of the SVZ (Fernando), and therefore decreases proliferation.

Still it is unknown whether $GABA_B$ receptors ($GABA_BRs$) are expressed in the SVZ. The high affinity GABA transporter GAT4 tightly regulates GABA levels in stem cells, but not in neuroblasts. Pharmacological inhibition of $GABA_AR$ in organotypic cultures *in vivo* induce increased proliferation. Conversely, inhibition of GAT4 produces the opposite effect (Liu et al., 2005).

In the SGZ, type I cells respond to tonic GABAergic stimulation (Tozuka et al., 2005; Ge et al., 2006). In the DG, γ2 subunit (presumably associated with the α5 subunit), has a unique role in maintaining adult precursor cells quiescence. Using a conditional transgenic γ2-KO mouse, Song et al. (2012a,b) found increases in proliferation and increases in symmetrical cell-renewal of type I cells. Interestingly, using an optogenetic approach they identify the source of GABAergic innervation in parvalbumin (PV) positive interneurons, but not somatostatin and vasoactive intestine polypeptide interneurons (Song et al., 2012a). Furthermore, α4 -KO mice showed an increase in proliferation as assessed using BrdU injection (Duveau et al., 2011). A mechanism by which GABA regulates proliferation is through regulation of epigenetic mechanisms that inhibits DNA synthesis (Fernando et al., 2011).

Together these results suggest that at least two distinct $GABA_AR$ subtypes control stem cells and neural precursor cells.

The role of $GABA_BR$ in type I cells was investigated by Felice et al. (2012). After blocking $GABA_BR$ with its antagonist

Table 2 | Comparison of the role of different GABA receptor and receptor subunits in different stages of mouse adult neurogenesis in the OB and DG.

		Gaba receptor	Model	Effect	References
PROLIFERATION	OB	$GABA_AR$	Pharmacological inhibition	Increase in proliferation	Liu et al., 2005
	DG	γ2	Conditional KO mice	Decrease in proliferation of type I cells	Song et al., 2012b
		α4	KO mice	Increase in cell proliferation	Duveau et al., 2011
		$GABA_B1$	Pharmacological blocking and transgenic KO mouse	Increase in proliferation	Felice et al., 2012 Rakic, 2002
MIGRATION	OB	α4	KO mice	Delayed radial migration	Unpublished
	DG	α2	KO mice	Increased distance of migration	Duveau et al., 2011
		α4	KO mice	Impaired migration	Duveau et al., 2011
SPECIFICATION	OB	NA	NA	NA	
	DG	$GABA_AR$	Agonist	Increased number of new neurons	Tozuka et al., 2005
		γ2	Conditional deletion	Increased fraction of astrocytes	Song et al., 2012a
INTEGRATION	OB	α2	Transgenic mice and virus injection	Reduction of dendritic ramifications and spines	Pallotto et al., 2012
	DG	α2	KO mice	Reduction of dendritic branching in mature cells	Duveau et al., 2011
		α4	KO mice	Reduction of dendrite growth and branching	Duveau et al., 2011
PLASTICITY	OB	α2	Transgenic mice and virus injection	Unable to restore spine density after enrichment	Pallotto et al., 2012
	DG	NA	NA	NA	

NA, not available.

CGP52432, they found an increase in cell proliferation in the ventral hippocampus after 21 days of treatment, but not after acute treatment with the drug. $GABA_BR$ is made up by different subunits $GABA_B1$ and $GABA_B2$ (Gassmann and Bettler, 2012). Giachino et al. (2014) demonstrated that $GABA_B1$-KO mice show an increased adult progenitor proliferation accompanied with an unaltered cell survival. Since $GABA_BR$ are expressed not only by GCs, these effects may be a result of an indirect effect.

In summary, $GABA_AR$ activation decreases the proliferation rate in both OB and DG. GABA concentrations modulate the number of neuroblasts generated by precursor cells, suggesting a negative feedback mechanism to maintain the balance between proliferation and migration (Bordey, 2007 rev).

MIGRATION: GABA DRIVES NEUROBLASTS MIGRATION

OB neuroblasts migrate a long distance, from the SVZ to the OB. They migrate tangentially to the SVZ and once within the OB, they migrate radially respect to the OB using a vascular scaffold (Bovetti et al., 2007). Many factors are involved in this process, and GABA seems to be an important modulator in the tangential migration process. Migrating neuroblasts in the RMS express a variety of $GABA_ARs$ in developmently-related combinations (Pathania et al., 2010). Ambient GABA in the RMS reduces the speed of neuroblast migration in acute slices of adult and juvenile mice. Blocking GABA transporters or enhancing GABA release from neuroblasts slows the speed of migration (Bolteus and Bordey, 2004). The migration speed is also regulated by the depolarizing effect of GABA. Mejia-Gervacio et al. (2011) silenced NKCC1 expression with a short harpin RNA strategy in OB acute slices, which did not allow them to make GABA depolarizing. They reported that NKCC1 activity is necessary for maintaining normal migratory speed and regulating the resting membrane potential of postnatal migratory neuroblasts. They also demonstrated that NKCC1 function is strongly reduced at the time in which the cells reach the GCL (Mejia-Gervacio et al., 2011). Conversely, in α4-KO mice, many neuroblasts that complete their tangential migration apparently fail to enter into the GCL, suggesting a role of tonic GABAergic transmission for regulating migration into the gray matter (Fritschy, unpublished).

In the DG, neuroblasts migrate only few microns from the neurogenic niche to their final location, and this process has consequently received less scrutiny. However, Duveau et al. (2011) showed that in α4-KO mice neuroblasts migrate a significantly shorter distance compared to wild type mice. Conversely an opposite phenotype was observed in α2-KO mice, where a higher number of neuroblasts migrate deeper into the GCL. In this example, GABA signaling has a dual and opposite role depending

on the receptor it acts on. This suggests that the intracellular pathway activated by the two different receptors may contribute to different functions. Moreover, activation of α4 subunit is critical modulating entry of neuroblasts into the GCL in both the DG and the OB.

DIFFERENTIATION

In the OB, it has been estimated that 50–75% of neuroblasts become GABAergic OB-GCs, while the remaining become PGCs. The fate of OB interneurons depends on genetic (Kohwi et al., 2005; Waclaw et al., 2006; Saino-Saito et al., 2007), spatial and temporal factors (for a review see Lledo et al., 2006). The role of $GABA_AR$-mediated transmission has yet to be investigated.

GABA is one of the major extrinsic factors regulating differentiation of Type II cells of the DG through $GABA_AR$. Indeed, administration of a $GABA_AR$ agonist significantly increased the number of new neurons labeled with BrdU, while the $GABA_A$ antagonist has no effect. Progenitors in the SGZ receive GABAergic, but not glutamatergic inputs. GABA induces the expression of NeuroD, a transcription factor that positively regulates neuronal differentiation toward DG-GCs (Tozuka et al., 2005).

Depolarizing action of GABA

One open question that has not been elucidated is the timing of when GABA switches from a depolarizing to a hyperpolarizing agent in adult-born neurons.

Using gramicidin perforated patch clump, Wang et al. (2003) demonstrated that in neuronal progenitors in the SVZ as well as migrating neuroblasts in the SVZ GABA has a depolarizing effect. Mejia-Gervacio et al. (2011) silenced NKCC1 expression with short a harpin RNA strategy in OB acute slices, to make GABA depolarizing. Although these data suggest a depolarizing role for GABA in perinatal neuroblast migration, it is still not clear when GABA switches from a depolarizing to a hyperpolarizing role during OB adult neurogenesis.

In the DG it is well established that GABA has a depolarizing effect on adult-born dentate GCs, recapitulating what happens during the ontogenic development. Ge et al. (2006) used a short harpin RNA strategy to silence NKCC1 activity. They found that the switch from GABA-induced depolarization occurs between 14 and 28 dpi (days post injection). More recently, Chancey et al. (2013) discovered that GABA depolarization is needed for AMPA receptor (AMPAR) synaptic incorporation in developing adult-born GCs.

INTEGRATION AND MATURATION

Many studies have described the development and integration of adult born neurons. Similarly, many reviews have been written to summarize these findings, for an in-depth analysis (see Petreanu and Alvarez-Buylla, 2002; van Praag et al., 2002; Carleton et al., 2003; Lledo et al., 2006; Kelsch et al., 2008, 2010; Dieni et al., 2012; Gu et al., 2013; Platel and Kelsch, 2013). Here, we want to review the role of $GABA_AR$ and $GABA_AR$ subunits in modulating integration and maturation of adult born neurons.

Structural development of adult-born neurons in the OB

eGFP lentiviral injection into the RMS to birth-date adult-born OB-GCs, were used to described GCs development (Petreanu and Alvarez-Buylla, 2002; Carleton et al., 2003; Pallotto et al., 2012). When neuroblasts arrive into the OB after having migrated along the RMS, they exhibit the typical bipolar morphology of migrating cells. According to Alvarez-Buylla classification, we can distinguish 5 different classes of adult born neurons according to their morphology. Class 1 cells are neuroblasts migrating tangentially toward the OB (observed 2–7 days after virus injection—dpi). Class 2 neurons leave the RMS and migrate radially in the OB (5–7 dpi). Class 3 neurons extend a simple apical dendrite toward the mitral cell layer (MCL) (9–13 dpi). In class 4 neurons, the apical dendrite has crossed the MCL and starts branching within the EPL (11–22 dpi). Finally, class neurons 5 are considered morphologically mature GCs with spiny apical dendrites branched in the EPL (from 15 dpi) (Alvarez-Buylla et al., 2001).

Similarly, adult-born GCs have a unique sequence of electrophysiological maturation. Migratory cells (stages 1 and 2) have membrane properties similar to immature precursors, and do not show spontaneous postsynaptic currents. In contrast, stages 3–5 neurons start to show excitatory and inhibitory postsynaptic currents (Carleton et al., 2003).

Pallotto et al. further described the maturation of adult-born GCs. Dendritic growth and ramification was monitored from 7 to 90 dpi (Pallotto et al., 2012). Sholl analysis on developing adult-born OB-GCs demonstrates that the cells reach a maximum in dendritic branching after 30 dpi, thin value decreasing at 90 dpi. A similar pruning has been shown also for dendritic spines. It has been reported that a maximum dendritic spine density in the EPL at 28 dpi when injecting in the SVZ and at 30 dpi when injecting in the RMS (Whitman and Greer, 2007; Pallotto et al., 2012). Similarly, adult-born PGCs undergo dendritic spine pruning, reaching the maximum spine density between 1 and 3 months post-injection (Livneh and Mizrahi, 2011). These authors suggest that dendritic structure is determined by animal age rather than neuronal age. Altogether these findings demonstrate that the integration of adult-born OB interneurons is a long process and that external factors play a role in shaping the adult morphology of the cell through a pruning mechanism.

Using the Cre-lox system to selectively silence α2-$GABA_AR$ in virally-transfected cells, Pallotto et al. (2012) investigated the role of phasic GABAergic transmission into adult-born OB-GCs. Inactivation of the α2 subunit gene has detrimental effects on adult-born GCs structural maturation. The authors showed that α2-KO cells have reduced dendritic branching and a number of reduced spines when compared to the WTs. Therefore, the presence of postsynaptic α2-$GABA_ARs$ is fundamental for the growth of dendrites and spines observed in WT mice.

Synaptic development in adult-born neurons in the OB

Adult-born OB-GCs are rapidly targeted by axon terminals as soon as they reach their final position in the GCL (Whitman and Greer, 2007; Kelsch et al., 2008; Panzanelli et al., 2009). GABAergic and glutamatergic contacts form on the dendrites and cell bodies within 3 dpi in the RMS and on apical dendrites only 1 day later, as shown by IHC quantification of synaptic puncta for

inhibitory and excitatory synapse markers. In addition, these contacts are already functional, as shown by whole cell patch clamp recordings (Panzanelli et al., 2009).

Quantification of synaptic inputs onto newborn OB-GCs at early stages of development (3–7 dpi) indicates initially a higher fraction of gephyrin positive puncta then PDS95. At 7 dpi, PDS95 clusters were predominant, suggesting a slight delay in the formation of glutamatergic contacts on newborn GCs. Absence of phasic GABAergic inputs though removal of the α2 subunit led to a marked reduction in spontaneous and evoked inhibitory post-synaptic currents (IPSCs). At the molecular level, the loss of α2 subunit is followed by a strong reduction of its scaffolding protein gephyrin. Presynaptic terminals were not affected and no compensatory effects by α3-$GABA_A$Rs, also expressed by OB-GCs, were evident. The reduced synaptic GABAergic function inputs also affected the development of glutamatergic contacts. After deletion of α2 subunit in adult-born GCs, Pallotto et al. (2012) found a reduction of glutamatergic synapses demonstrated by a decrease in spontaneous excitatory postsynaptic currents (EPSCs), and a reduction in the PDS95 positive puncta on the spine head.

Development of adult-born neurons in the DG

Within 4 weeks after symmetric division, newborn DG-GCs extend their dendrites into the molecular layer, they first receive slow GABAergic inputs from hilar interneurons and from Ivy cells (Deshpande et al., 2013). Later, they receive numerous glutamatergic inputs from lateral and medial perforant pathways, and lastly perisomatic GABA synapses from various types of basket cells [parvalbumin or cholecystokinin- expressing cells (Katona et al., 1999; Song et al., 2013)], as well as axo-axonic contacts from chandelier cells. Adult-born GC axon projections reach the *stratum lucidum* of the CA3 region as well as the *stratum oriens*, where they form mossy fiber terminals (Esposito et al., 2005; Overstreet Wadiche et al., 2005; Toni et al., 2007; Jessberger et al., 2008; Markwardt and Overstreet-Wadiche, 2008; Zhao et al., 2010). From recent literature it is emerging that another important role of GABA neurotransmission is in the development of DG-GCs. Since GCs express different $GABA_A$R subunits, Duveau et al. (2011) dissected the role of α2– and α4-containing $GABA_A$R using lentivirus injection in KO mice. At 14 dpi α4-KO mice show a significant reduction of dendritic ramification, whereas the initial growth of dendrites was normal in α2- and δ-KO. At later stages (42 dpi) also α2-KO has a reduced dendritic complexity suggesting that the two different $GABA_A$R subunits have different roles in the dendritic tree development. However, it is important to note that DG-GCs also receive phasic inhibition by α1-$GABA_A$R (Sun et al., 2004), therefore, the deletion of the α2 subunit may be compensated for, and may not cause a complete loss of GABAergic synaptic inputs onto adult-born GCs.

PLASTICITY AND CRITICAL PERIOD

Adult-born GC development described above is not only regulated by an intrinsic program or local signaling molecules. The behavior of the neurons is also affected by their cellular age and by changes in the local environment. The term "critical period" is widely used to describe a specific time window in which neuronal proprieties are particularly prone to modification by external stimuli or experience. Consequences of the critical period are an enhanced morphological and synaptic plasticity that may shape behaviors.

Nissant et al. (2009) demonstrated the tendency of adult-born OB-GCs to undergo long-term potentiation (LTP) after their arrival in the bulb. The ability to undergo LTP faded as newborn neurons matured. LTP is the leading candidate mechanism for memory encoding and the presence of LTP only in a defined "time window" (around 20–30 dpi) suggests that newborn GCs are particularly sensitive to synaptic plasticity (Nissant et al., 2009). External stimuli shape the final morphology of OB-GCs acting on synaptic connectivity. Pallotto et al. (2012) documented that adult-born GCs that were subjected to odor enrichment showed increased spine density. This indicates that by controlling odor exposure during a "critical period," it is possible to control the level of excitatory drive onto GCs trough principal cell activation. The increase in spine density is most likely due to a stabilization of synaptic turnover rate (Livneh and Mizrahi, 2012).

Varying the degree of sensory inputs to the OB, using olfactory enriched environment or depriving sensory stimuli, Pallotto et al. (2012) found that none of the two treatments caused significant changes in spine density in adult-born OB-GCs lacking the α2-$GABA_A$R subunit. This observation indicates that GABAergic synaptic transmission mediated by α2-$GABA_A$Rs is required for structural adaptations of adult-born GCs in response to sensory challenges during the phase of dendritic/spine exuberance. GABA neurotransmission may be a candidate to regulate the opening/closing of the critical period in adult neurogenesis in the OB.

Between 28 and 42 days after birth, adult-born DG-GCs show a critical period with enhanced LTP (Ge et al., 2007a; Marin-Burgin et al., 2012). In this time window, they have different mechanisms that make them hypersensitive to stimuli. For example, adult-born GCs have lower activation threshold due to an enhanced E/I balance (Marin-Burgin et al., 2012). Furthermore, adult-born DG-GCs express NR2B NMDA receptor (NMDAR) subunit that appears to be associated with enhanced plasticity (Ge et al., 2007a).

Interestingly, the critical period is preceded by the transition of GABA from excitatory to inhibitory (Ge et al., 2006). Recently, Chancey et al. (2013) thoroughly investigated the role of GABAergic depolarization during the critical period. They found that GABAergic synaptic depolarization enables activation of NMDAR in the absence of AMPAR-mediated transmission *in vitro* as well as *in vivo* after a brief exposure to enriched environment. Therefore, GABAergic depolarization is required and allows excitatory synapse un-silencing that is induced by activity.

SURVIVAL

Both in OB and DG, a massive number of adult-born neurons undergo programmed cell death within a month after birth [specifically, 50% in the OB (Petreanu and Alvarez-Buylla, 2002) and 60–80% in the DG (Cameron and McKay, 2001)]. While deletion of some $GABA_A$R subunits affect morphology and plasticity of adult-born cells (see previous paragraphs), it does not explicitly affect survival. This raises the question: is morphological

maturation unrelated to survival? According to Pallotto, Duveau and Giachino deletion of different $GABA_AR$ and $GABA_BR$ subunits do not affect neuroblasts survival (Rakic, 2002; Duveau et al., 2011; Pallotto et al., 2012). However, up to now, few studies explored properly the role of GABA signaling in the survival of adult-born cells. It would be interesting to use specific markers, like caspase or ssDNA antibodies, to detect the cell death rate in adult-born neurons in OB and DG in mice lacking specific subunits of $GABA_AR$.

BEYOND GABAergic SIGNALING

The formation of a functional network in the CNS requires cell integration, synapse formation and maturation through the orchestration of several factors. Understanding these processes is a major challenge in the neuroscience field. Adult neurogenesis is a formidable tool for this purpose. Adult-born cells are rapidly targeted by axon terminals, forming functional excitatory and inhibitory contacts. Considering that during the first month of development, about half of the adult-born cells die (Cameron and McKay, 2001; Winner et al., 2002), synapse formation could represent a way by which GCs are positively selected. Soon after the initial integration step, adult-born GCs go through a period of prominent structural reorganization involving changes in dendritic arborization and spine density (Whitman and Greer, 2007; Livneh and Mizrahi, 2011; Pallotto et al., 2012) (**Figure 2**).

In both OB and DG, adult-born neurons show a time window in which adult-born neurons are more susceptible to stimuli, showing an enhanced LTP (**Figure 2**). In the OB, absence of phasic GABAergic input, mediated by α2-$GABA_ARs$, makes adult-born GCs incapable of responding to external stimuli. In the DG, GABAergic activity allows synapse unsilencing driving AMPAR insertion (Chancey et al., 2013). However, the molecular mechanism by which GABA exerts its function is still not clear.

If there is a huge variety of $GABA_AR$ each of them modulating a different function in adult neurogenesis, then there must be heterogeneous downstream pathways. Here we review three pathways that have been shown to be involved in the downstream molecular signaling. Those pathways may be independent or they may converge and interact at any level.

SECOND MESSENGERS: EXAMPLES FROM CREB AND PI3K SIGNALING PATHWAY

Another common mechanism in the OB and DG by which GABAergic transmission affect maturation and the critical period of adult-born neurons could be the cAMP response element-binding protein (CREB) pathway (Merz et al., 2011). CREB is a transcription factor involved in many different aspects of adult neurogenesis (Lonze and Ginty, 2002). CREB and its active form, pCREB is expressed in the SVZ and by adult-born neurons in the OB. pCREB is only sporadically expressed by DCX^+ neuroblasts in the SVZ, but is strongly present in migrating neuroblasts in the RMS and OB (Herold et al., 2011). The loss of CREB function results in a reduction of the survival rate of newborn neurons and impairs morphological differentiation (Giachino et al., 2005; Herold et al., 2011). Similarly, in the DG the timing of pCREB expression is highly regulated and present in DCX+ cells. In a cell autonomous manner, CREB signaling pathway regulates survival and morphological development (Jagasia et al., 2009).

pCREB controls multiple steps including proliferation, survival, neurite outgrowth and dendrite branching. Formation of spines accompanied with glutamatergic inputs occurs at later stage (28 days after birth), suggesting that GABA might trigger the signaling cascade leading to the phosphorylation of CREB (Magill et al., 2010).

An interesting link between GABAergic activity and CREB signaling come from the work of Jagasia et al. In this work, using an *in vivo* approach with retroviral injection in the DG, the authors demonstrate that CREB phosphorylation and signaling affect survival and maturation and integration of adult born DG-GCs. The peak of CREB phosphorylation occurs when GABA acts with a depolarizing effect on adult born GCs. Using a shNKCC1 virus to ablate the depolarizing effect of GABA, the authors showed that impairment in morphological maturation and differentiation and a reduced survival rate can be rescued by CREB activation (Jagasia et al., 2009).

shRNA against γ2 subunit in adult-born DG-GCs causes a reduced dendritic growth. A similar phenotype is also seen in mice where NKCC1 is down regulated by shRNA (Kim et al., 2012). The selective deletion of the α2 and α4 - subunit-containing $GABA_ARs$ in adult-born -GCs, alters dendritogenesis but not survival suggesting that there might be another $GABA_AR$ involved in CREB phosphorylation during the differentiation of adult-born neurons. This different $GABA_AR$ subunit may regulate the survival but not the development or integration of adult born GCs (Duveau et al., 2011). Despite the lack of strong evidences of $GABA_AR$ involvement in survival, this phenomenon is not deeply studied. Therefore, we cannot exclude a role of other $GABA_AR$ subunits having a role in survival of adult-born neurons.

Epigenetic modifications elicited by $GABA_AR$ activation also influence cell proliferation in the SVZ (Fernando et al., 2011). An interesting paper from Fernando et al. showed *in vitro* and *in vivo* that acute pharmacological modulation of $GABA_AR$ with muscimol or bicuculline leads to increase or decrease of histone H2AX, respectively. As a consequence, the authors observed a decrease or an increase of BrDU incorporation from type C and B cells of the SVZ. Pharmacological modulation of $GABA_AR$, therefore affects proliferation. This effect is also observed long-term after chronic pharmacological treatment, and it affects the number of BrDU positive GCs in the GCL. In a previous paper from the same group, it was demonstrated in embryonic stem cells that $GABA_AR$ signals through phosphatidylinositol-3-OH kineases to phosphorylate the histone variant H2AX (Andang et al., 2008). Together, these works indicate that $GABA_AR$ modulate proliferation thought an epigenetic mechanism, that may have important consequence for long-term modulation of the neural niche size and composition, and therefore of the adult neuronal cells that are produced.

EXTRASYNAPTIC RECEPTORS MEDIATE THEIR EFFECTS VIA CA^{2+} SIGNALING

E/I balance has a fundamental role in neuron development. Alterations of the E/I balance cause defects such as impairment

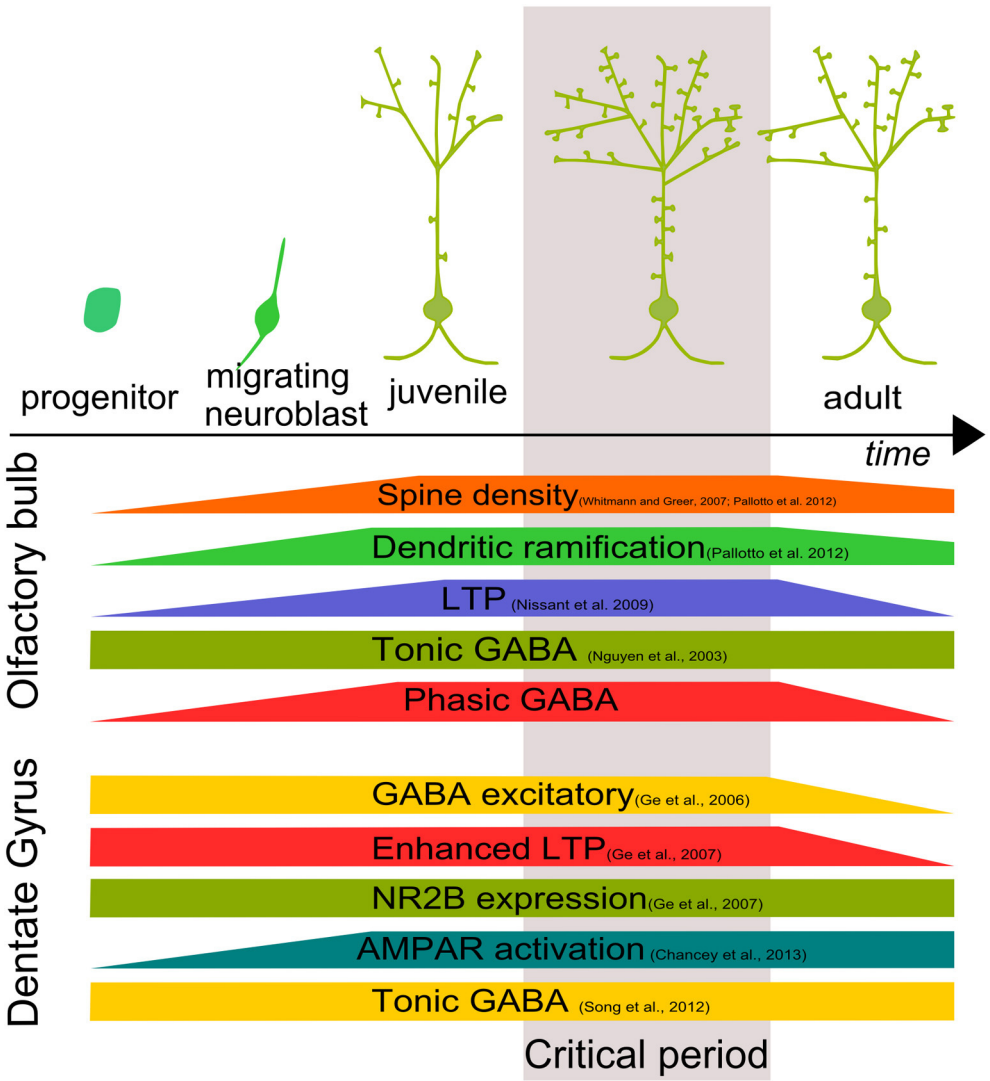

FIGURE 2 | Development and critical period of adult-born neurons. Adult–born GC growth is characterized by a time window in which morphological, biochemical and physiological characteristics are finely tuned both in the OB and DG defining a "critical period" for adult-born cell development.

of dendritic growth during both in the juvenile and mature development (Cancedda et al., 2007; Ge et al., 2007b). During development, when GABA has an excitatory role, GABAergic-mediated Ca^{2+} signaling mediates several aspects such as migration and maturation. GABA neurotransmitter increase $[Ca_i^{2+}]$ influx thought voltage-sensitive channels (Yuste and Katz, 1991). In cortical development, chemotaxis and chemokinesis are mediated by an increase in intracellular Ca^{2+} concentration stimulated by GABA (Behar et al., 1996). In type A progenitor SVZ cells, $GABA_AR$ activation induce variation in $[Ca_i^{2+}]$ to modulate proliferation (Nguyen et al., 2003). Similarly, in SVZ-derived neurons, an exposure of 10–30 s to GABA induces $[Ca_i^{2+}]$ increase. $GABA_AR$ activation is dependent of L-type voltage-gated Ca^{2+} channels (Gascon et al., 2006). In developing DG-GCs, GABA induces Ca^{2+} transients via L-type Ca^{2+} channels. These Ca^{2+} transients are important for suppressing axonal but not dendritic growth (Lee et al., 2012). In the adult brain, spine shrinkage and elimination are promoted by activation of $GABA_AR$ occurring after an action potential. In this particular case, GABAergic inhibition suppresses local dendritic Ca^{2+} transient that promotes competitive selection of dendritic spines (Hayama et al., 2013). In the DG, GABAergic hippocampal activity depolarizes type-2 cells leading to an increase in intracellular Ca^{2+} concentration and promoting activity-dependent neuronal differentiation. In the SVZ, $GABA_AR$ induces depolarization leading to the opening of L-type Ca^{2+} channels (Young et al., 2010). In SVZ precursor cells, the presence of tonic currents has been reported (Liu et al., 2005; Bordey, 2007). These data support the notion that Ca^{2+} dynamics are regulated by tonic $GABA_AR$ activity at early stage of neuronal development; however phasic GABAergic activation cannot be excluded.

SYNAPTIC RECEPTORS AND GEPHYRIN SIGNAL

GABAergic signals are mediated by both synaptic and extrasynaptic receptors. The presence of synaptic and extrasynaptic $GABA_A$Rs on the plasma membrane is a highly dynamic state is and regulated by multiple mechanisms influencing the position and properties of the receptor, including interactions with the gephyrin scaffold. Gephyrin mostly regulates the clustering of synaptic $GABA_A$Rs. Gephyrin is crucial for the formation of GABAergic synapses, but it also interacts with other signaling molecules. $GABA_A$R-gephyrin interactions regulate gephyrin's clustering properties and/or are anchored at GABAergic synapses by binding to gephyrin. *In vitro* work emphasizes the importance of gephyrin phosphorylation in regulainge GABAergic synaptic function (Tyagarajan and Fritschy, 2010; Tyagarajan et al., 2011, 2013). In particular, inhibiting phosphorylation of the residue Ser270 of gephyrin leads to an upregulation of postsynaptic gephyrin clusters, and consequently to an increase in the frequency and amplitude of mini GABAergic currents (Tyagarajan et al., 2011). Conversely, a mutation in a surface-exposed loop (L2B) prevents gephyrin from clustering and prevents the formation of GABAergic postsynaptic densities (Lardi-Studler et al., 2007). This suggests that the ability of gephyrin in modulating GABAergic synaptogenesis can have a direct influence on the stabilization of $GABA_A$Rs in a phosphorylation-dependent manner or on downstream signaling cascades. Downstream gephyrin signaling involves adhesion molecules such as neuroligin 2, or GDP/GTP exchange factors (GEFs) such as collybistin, and also small Rho- GTPases as Cdc-42 or profiling (for a review see Vadodaria and Jessberger, 2013). Cdc-42 can modulate actin and microtubules. This signaling may play a role in maturation and plasticity of adult-born GCs by interacting with the cytoskeleton. Therefore, the morphological deficits seen in adult-born OB-GCs in α2-KO might be due to the fact that gephyrin clusters are disrupted (Pallotto et al., 2012). This presumably leads to a dispersion of signaling molecules such as collybistin and Cdc-42 away from the synapses. In particular, this possibility implies that the reduction of spine density and dendritic growth in adult-born OB-GCs, which has been observed in Pallotto et al. (2012) after selective depletion of α2 subunit, might be due to impaired gephyrin clustering (Pallotto et al., 2012).

To better understand the role of the downstream signal pathway of gephyrin, and therefore its role as scaffold for a proper adult-born cell maturation and integration, it would be interesting to study its different states of phosphorylation and expression patterns.

In summary, GABAergic activity exerts a variety of functions in adult neurogenesis. The role of the neurotransmitter is defined by a variety of $GABA_A$R subunits. Sometimes, two different $GABA_A$R subunits exert different and opposite effects. For example in the DG, the α2 and α4 subunits have an opposing effect on cell migration (Duveau et al., 2011) (for a comparison see **Table 1**). Multiple pathways may mediate the specific effects of the different subunits. Here, we have only described a subset of these molecular pathways, which are intermingled, making it difficult to determine their specific roles. For example, it is possible that GABA induces influx of Ca^{2+} in newborn GCs leading to CREB induced gene expression. Similarly, Ca^{2+} may promote gephyrin phosphorylation. It would be interesting to understand how GABAergic synaptic and extrasynaptic receptor activity is orchestrated. Although it is known that different $GABA_A$R subtypes modulate different aspects of adult neurogenesis, which downstream pathways are involved is still unclear.

Adult neurogenesis provides neuroblasts in a mature network. It is a unique feature that neuroblasts have to integrate into already existing network, lacking all the neurotrophic factors that are present during brain development. We still need to understand which mechanisms and which receptors GABA used to modulate the intracellular pathways that leads neuroblasts to integrate and mature. Beyond the fascinating quest to understand the mechanisms that drive adult neurogenesis, unraveling this issue will help us better understand not only brain function and development but also neurodevelopmental disorders. This may contribute to new strategies for cell replacement therapies.

ACKNOWLEDGMENTS

We would like to thank Dr. Jean-Marc Fritschy (University of Zurich) for discussion and comments on the manuscript. This research was supported by the Intramural research program of the NIH, NINDS and the Swiss National Science Foundation. We are also grateful to Dr. Paul Watkins and Dr. Christopher Harris for corrections to the English.

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

Received: 03 March 2014; accepted: 02 June 2014; published online: 20 June 2014.
Citation: Pallotto M and Deprez F (2014) Regulation of adult neurogenesis by GABAergic transmission: signaling beyond $GABA_A$-receptors. Front. Cell. Neurosci. ***8****:166. doi: 10.3389/fncel.2014.00166*
This article was submitted to the journal Frontiers in Cellular Neuroscience.

frontiers in
CELLULAR NEUROSCIENCE

REVIEW ARTICLE
published: 17 June 2014
doi: 10.3389/fncel.2014.00161

Structure, function, and plasticity of GABA transporters

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GABA transporters belong to a large family of neurotransmitter:sodium symporters. They are widely expressed throughout the brain, with different levels of expression in different brain regions. GABA transporters are present in neurons and in astrocytes and their activity is crucial to regulate the extracellular concentration of GABA under basal conditions and during ongoing synaptic events. Numerous efforts have been devoted to determine the structural and functional properties of GABA transporters. There is also evidence that the expression of GABA transporters on the cell membrane and their lateral mobility can be modulated by different intracellular signaling cascades. The strength of individual synaptic contacts and the activity of entire neuronal networks may be finely tuned by altering the density, distribution and diffusion rate of GABA transporters within the cell membrane. These findings are intriguing because they suggest the existence of complex regulatory systems that control the plasticity of GABAergic transmission in the brain. Here we review the current knowledge on the structural and functional properties of GABA transporters and highlight the molecular mechanisms that alter the expression and mobility of GABA transporters at central synapses.

Keywords: GABA, GABA transporters, uptake, synaptic transmission, synaptic plasticity, GAT1, GAT3, SLC6

INTRODUCTION

The brain utilizes GABAergic synaptic transmission to modulate the ongoing activity of neuronal networks. By acting on ionotropic and metabotropic receptors, GABA controls the generation of membrane potential oscillations, the time window over which synaptic inputs are integrated and the temporal structure of the activity patterns produced by entire populations of neurons (Pouille and Scanziani, 2001; Hajos et al., 2004; Akam and Kullmann, 2010; Mann and Mody, 2010; Stark et al., 2013). These actions require a fine control of the timing of GABA receptor activation which, in turn, depends on the precise timing of GABA release from pre-synaptic terminals and GABA clearance from the extracellular space. Extracellular GABA is not subject to enzymatic breakdown, and its clearance relies entirely on diffusion and uptake by specific transporters. GABA transporters belong to a large family of neurotransmitter:sodium symporters, and are widely expressed throughout the brain. GABA transporters are expressed in different cell types, including neurons and astrocytes, at expression levels that vary across different brain regions. Experimental evidence indicates that the distribution of GABA transporters in the cell membrane is highly dynamic and can be modified in an activity-dependent manner. For example, there are intracellular signaling cascades that regulate the cytoplasm-to-surface partitioning of GABA transporters (Corey et al., 1994; Whitworth and Quick, 2001) and the interaction between GABA transporters and components of the cytoskeleton, which control the mobility of these molecules within the cell membrane (Imoukhuede et al., 2009). In this review, we provide an overview of the structural and functional properties of GABA transporters and of the molecular mechanisms that can alter their expression and mobility in the cell membrane.

THE GABA TRANSPORTER FAMILY AND THE GABA TRANSPORTER GROUP

GABA transporters belong to a family of neurotransmitter:sodium symporters that in humans is referred to as the solute carrier 6 (SLC6) family. The SLC6 family is composed of 20 members and, based on sequence composition, it is subdivided into four groups including GABA, osmolyte and creatine transporters (**Figure 1**, *blue section*), neurotransmitter amino acid (**Figure 1**, *pink section*), monoamine (**Figure 1**, *green section*) and nutrient amino acid/orphan transporters (**Figure 1**, *gray section*). The currently accepted nomenclature that is used to identify members of the SLC6 family represents the nomenclature of GABA transporters in humans (A1-20) and rats (GAT, BGT, NTT, etc.). The nomenclature of GABA transporters in mice is different and somewhat confusing when compared to the ones mentioned above. In mice, GAT2 corresponds to A12/BGT1, GAT3 corresponds to A13/GAT2 and GAT4 corresponds to A11/GAT3, while GAT1 carries the same name as in humans and rats (A1) (Nelson, 1998; Cohen-Kfir et al., 2005). In this review, for simplicity, we use the human/rat GABA transporter nomenclature. There is overlapping substrate specificity across all four groups of transporters within the SLC6 family. For example, betaine is transported by A12/BGT1 and A20/SIT1, and A9/GlyT1, A5/GlyT2, A19/B^0AT1, A18/B^0AT3, A14/$ATB^{0,+}$ all transport glycine across the cell membrane (Broer and Gether, 2012). There is also overlapping substrate specificity within each group of the SLC6 family. For example, the monoamine transporters have low selectivity for decarboxylated derivatives of aromatic aminoacids like tyrosine and triptophane. Accordingly, the dopamine transporter A3/DAT also transports noradrenaline (Broer and Gether, 2012), the noradrenaline transporter A2/NET also transports dopamine

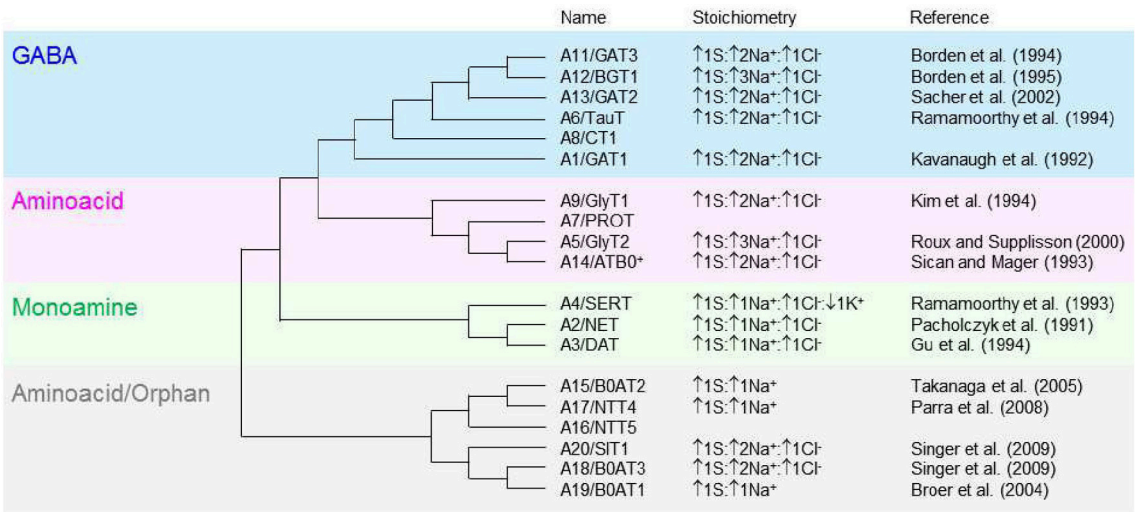

FIGURE 1 | Molecular phylogenetic analysis of the SLC6 neurotransmitter transporter family in *Homo sapiens*. The SLC6 family is divided into four groups, including the GABA (blue), aminoacid (pink), monoamine (green) and aminoacid/orphan transporters (gray). The evolutionary history is inferred by using the maximum likelihood method based on the JTT matrix-based model (Jones et al., 1992). The initial tree for the heuristic search is obtained automatically by applying neighbor-join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. The analysis is performed on 19 aminoacid sequences because the gene encoding the SLC6A10 transporter is thought to be a pseudogene (Kristensen et al., 2011). All positions containing gaps and missing data were eliminated. There is a total of 372 positions in the final dataset. The evolutionary analysis is obtained with MEGA5 (Tamura et al., 2011). The tree is not drawn to scale; it includes the SLC and the commonly used neurotransmitter transporter nomenclature. The stoichiometry and direction of the transport cycle are included, when known, together with the corresponding reference.

(Gether et al., 2006) and the serotonin transporter A4/SERT also transports dopamine, albeit with low-affinity (Larsen et al., 2011).

The GABA transporters group comprises six transporters: A1/GAT1, A13/GAT2, A11/GAT3, A12/BGT1, A8/CT1, and A6/TauT. All members can transport GABA and other molecules and there is overlapping substrate specificity also within the GABA transporters group. For example, GAT2 and GAT3 accept substrates with a carboxyl group in the β-position and an amino group in the γ-position of their carbon backbone structure, like GABA and β-alanine. Conversely, taurine, which has a sulphonate—not a carboxyl—group in the β-position, is only transported by TauT; creatine, in which the amino group in the γ-position is part of the guanidino group, is only transported by CT1; betaine, in which this group is methylated, is only transported by BGT1. GAT1, GAT2, and GAT3 (particularly GAT1) are the most extensively characterized GABA transporters and will be the main focus of discussion in this review.

THE BIOPHYSICAL PROPERTIES

The early experiments involving solubilization of GABA transporters from rat brains and subsequent reconstitution in proteoliposomes revealed four important biophysical properties of GABA transporters (Kanner, 1978). *First,* they showed that GABA transport is an active process, not directly coupled to ATP hydrolysis but requiring the presence of an inward electrochemical gradient for Na^+, typically created by the membrane Na^+/K^+ ATPase. *Second,* GABA transporters have low micromolar, steady-state affinity for GABA, with a Michaelis-Menten constant of 2.5 μM [later found to be 3.1–10.6 μM for GAT1 (Guastella et al., 1990)]. *Third,* replacement experiments in which Li^+, NH_{4+}, $Tris^+$, K^+ were used as substitutes for Na^+ showed that GABA transport is only supported by Na^+, not by any of the other cations (see also Iversen and Neal, 1968). *Fourth,* the increase in GABA uptake observed in the presence of the K^+-selective ionophore valinomycin indicated that GABA transport is electrogenic (and voltage-dependent) (Kanner, 1978). These fundamental aspects of GABA transporter function were all confirmed when GABA transporters were purified (Radian and Kanner, 1985) and identified (Radian et al., 1986) and when the first member of the GABA transporter subgroup, GAT1, was cloned (Guastella et al., 1990). Kinetic and thermodynamic experiments indicate that, in addition to Na^+, extracellular Cl^- is also required for GABA transport. These findings are thought to be consistent with the existence of a co-transport mechanism for GABA, Na^+ and Cl^- by GABA transporters. Accordingly, the most accredited stoichiometry of mammalian GABA transporters is $1GABA:2Na^+:1Cl^-$. Since GABA is a zwitterionic molecule, this stoichiometry leads to a net influx of one positive charge per transport cycle (Radian and Kanner, 1983; Kavanaugh et al., 1992; Mager et al., 1993; Lu and Hilgemann, 1999b). In some radioactive tracer flux experiments, however, a net charge influx of two positive charges per transport cycle has been measured (Pastuszko et al., 1982; Loo et al., 2000). Because activation of GABA transporters triggered simultaneous influx and efflux of Cl^- (Loo et al., 2000), it was proposed that there is an exchange mechanism that couples Cl^- influx and efflux across the cell membrane. If this were the case, the proposed stoichiometry of $1GABA:2Na^+:1Cl^-$ would only reflect the stoichiometry of

GABA influx, but the net stoichiometry for the entire transport cycle would be 1GABA:2Na^+. It has been reasoned that co-transporting Cl^- together with Na^+ and GABA would add very little energy to drive the uphill transport of GABA, because the reversal potential for Cl^- is close to the resting membrane potential (Loo et al., 2000). This reasoning obviously holds only if considering GABA transport in cellular or sub-cellular compartments where the reversal potential for Cl^- is hyperpolarized, at a time when the cell is not experiencing any depolarizing event (e.g., action potential firing, sub-threshold depolarization). The counter argument, however, is that the functional relevance of an electroneutral exchange of Cl^- across the cell membrane is unclear. Recent kinetics experiments indicate that Cl^- is only required for the generation of GABA-induced steady-state currents, not for GABA-induced pre-steady-state currents, which reflect the first electrogenic steps in the transport cycle (Bicho and Grewer, 2005). This result could be explained if Cl^- exchange occurred in a reaction step distinct from—but thermodynamically coupled to—the translocation of GABA and Na^+ across the membrane. The Cl^- exchange would occur while the transporter is in a conformational state that allows it to take up GABA and Na^+ (Bicho and Grewer, 2005). Cl^- binding to the extracellular side of GAT1 could facilitate intracellular GABA release from the transporter. Therefore the entire forward transport cycle would include rapid binding and translocation of GABA and Na^+, slower (12 ms) intracellular dissociation of GABA and Na^+ and rapid Cl^- exchange (Bicho and Grewer, 2005).

THE TURNOVER RATE OF GABA TRANSPORTERS

There are numerous and different estimates for the translocation rate of GABA and co-transported ions across the membrane via GAT1. For the forward mode (i.e., the mode that describes removal of GABA from the extracellular space toward the cytoplasm and transporter re-orientation in the cell membrane), the initial estimates at 22°C suggested a value of 2.5 s^{-1} in *Xenopus* oocytes (Radian et al., 1986). This value agrees well with the turnover rate found by others in the same preparation and in similar experimental conditions: 5.8–7.6 s^{-1} at −60 mV (Eckstein-Ludwig et al., 1999), 6.3 s^{-1} at −60 mV (Liu et al., 1998), 6–13 s^{-1} at −80 mV (Mager et al., 1993), 13 s^{-1} at −40 mV (Bicho and Grewer, 2005). However, other reports have also estimated turnover rates at 37°C and −50–90 mV of 73–93 s^{-1}, much higher than it would be predicted by correcting the previous values for the estimated Q_{10} value of 2.8 (Gonzales et al., 2007). This discrepancy may be attributed to methodological differences, as the latter estimates are obtained using correlative freeze-fracture and electrophysiology experiments. For the reverse mode (i.e., the mode that describes GABA release into the extracellular space and transporter re-orientation in the cell membrane), the available estimates suggest turnover rates of 3 s^{-1} at −120 mV and 60 s^{-1} at +120 mV at 33°C (Lu and Hilgemann, 1999a).

THE CURRENTS ASSOCIATED WITH GABA TRANSPORT

The initial biophysical characterization of GAT1 expressed heterologously in *Xenopus* oocytes did not provide an indication that GAT1 could generate any other current than the stoichiometric current described above (Hilgemann and Lu, 1999; Lu and Hilgemann, 1999a,b). Once GAT1 was expressed in HEK293 and HeLa cells, however, it became evident that GABA binding to GAT1 gates at least two more currents that are stoichiometrically uncoupled from the translocation of GABA, Na^+ and Cl^- across the membrane. The two stoichiometrically uncoupled currents are: (1) an agonist-induced Na^+ inward current (Risso et al., 1996); (2) an agonist-independent leak cationic current carried by alkali ions (Cammack and Schwartz, 1996). The lack of these currents in *Xenopus* oocytes may reflect different functional properties of GAT1 in *Xenopus* vs. mammalian expression systems (Lu and Hilgemann, 1999b). They may also be due to a technical limitation of *Xenopus* oocytes, where small Na^+ currents are not easily resolved (Lu and Hilgemann, 1999b). Even though a fully detailed, direct comparison between the agonist-induced stoichiometrically coupled and uncoupled currents in these different expression systems is not available, the Na^+-inward current could actually be significantly larger than one would expect based on the *Xenopus* oocytes studies. In fact, it has been suggested that this current could contribute 4-10 times more current than the stoichiometric component of GAT1 (Eckstein-Ludwig et al., 1999). Competitive GAT1 antagonists like tiagabine block both the stoichiometric and the Na^+ inward current with K_i values of 2 and 0.3 μM, respectively (Eckstein-Ludwig et al., 1999). In contrast, the GAT1 inhibitor SKF899A can be used to separate these two current components, because SKF89976A acts as a low-affinity, competitive antagonist of the stoichiometric current ($K_i = 7$ μM) and as a high-affinity, non-competitive antagonist for the Na^+ inward current [$K_i = 0.03$ μM (Krause and Schwarz, 2005)]. The contribution of the leak current to the total current generated by GAT1 is modest. This is in part due to the fact that its conductance is small [$g_{leak} = 0.36 \pm 0.18$ nS (Cammack and Schwartz, 1996)] and in part to the fact that this current is inhibited by intracellular Na^+ concentrations that are typically found in the cytosol of living cells ($K_i = 3$ mM) and often reproduced during electrophysiological patch-clamp recordings (Macaulay et al., 2002). The ability of GAT1 to act as a channel as well as a transporter is not an uncommon feature among neurotransmitter transporters: it is reminiscent of the mechanisms of action of glutamate transporters, which also generate agonist-induced stoichiometrically coupled and uncoupled currents (Wadiche et al., 1995). For both GABA and glutamate transporters, the stoichiometric current is inwardly directed. However, the agonist-induced stoichiometrically uncoupled current is cationic (and depolarizing) in GABA transporters and anionic (and often hyperpolarizing) in glutamate transporters. Activation of the stoichiometrically uncoupled, glutamate transporter anionic current in retinal rod bipolar cell terminals hyperpolarizes the cell membrane and inhibits neurotransmitter release (Veruki et al., 2006). The role of the stoichiometrically uncoupled, GABA transporter cationic current is not known but one hypothesis is that activation of this current at pre-synaptic inhibitory terminals could serve as a negative feedback mechanism that, by depolarizing the cell membrane potential, ultimately inhibits GABA uptake.

One other functional aspect of the agonist-induced stoichiometrically coupled and uncoupled currents that remains currently unknown is whether they share a common permeation pathway with the substrate or if, as proposed for analogous

currents generated by agonist binding to glutamate transporters, the two pathways are independent from one another (Ryan et al., 2004). Although this and other mechanistic questions about GABA transporters remain currently unanswered, the recent discovery of the crystal structure of $LeuT_{Aa}$, a bacterial homolog from *Aquifex aeolicus* (Yamashita et al., 2005) could allow for an unprecedented detailed level of understanding of this and other details about the transport process.

THE STRUCTURE OF A PROKARYOTIC GABA TRANSPORTER HOMOLOG

Aquifex aeolicus is a thermophilic bacterium that grows best at 95°C. The name refers to the fact that this bacterium produces water as a byproduct of its respiration (hence *Aquifex*) and was first isolated near underwater volcanic vents in the Aeolic Islands, north of Sicily (hence *aeolicus*). $LeuT_{Aa}$ is an experimentally tractable prokaryotic leucine transporter. It is evolutionary distant from GAT1 and shares 20–25% sequence similarity with GAT1 and other members of the SLC6 family including glycine, dopamine and serotonin transporters (Yamashita et al., 2005). The proposed stoichiometry of $LeuT_{Aa}$ (1 leucine:2 Na^+) indicates that substrate transport via $LeuT_{Aa}$ is not Cl^--dependent, in contrast to GAT1. Despite these differences, the structural conservation between $LeuT_{Aa}$ and other members of the SLC6 family is thought to be remarkably high (Abramson and Wright, 2009). This supports the usefulness and validity of $LeuT_{Aa}$ as a template model for the analysis of the structural architecture and function of transporters in the SLC6 family (Kristensen et al., 2011). $LeuT_{Aa}$ has a 70Å tall and 48Å wide cylindrical structure with 12 transmembrane domains (TM1-12), intracellular N- and C-terminal domains and extracellular, glycosylated regions (**Figure 2**), as previously deduced from the hydropathy analysis of the aminoacidic sequence of GAT1 (Guastella et al., 1990). $LeuT_{Aa}$ assembles as a dimer, with each protomer capable of independently binding and translocating leucine and the two co-transported Na^+ ions. According to FRET experiments, GAT1 also assembles as a multimeric structure (Schmid et al., 2001; Moss et al., 2009). Each monomer is capable of transporting GABA independently (Soragna et al., 2005), but the multimerization process allows trafficking of GAT1 from the endoplasmic reticulum to the plasma membrane (Farhan et al., 2006). The essential core structure of $LeuT_{Aa}$ is formed by TM1-10. TM11-12 participate in multimerization and the N- and C-terminal domains are not required for GABA transporter activity (Mabjeesh and Kanner, 1992). Consistent with these findings, other functional bacterial homologs of GAT1, like the transporter protein encoded by the tnaT gene of *Symbiobacterium thermophilum,* consist only of TM1-10 (Androutsellis-Theotokis et al., 2003). Some of the key residues in TM1-10 include: (1) Arg 69 (TM1), essential for substrate transport (Pantanowitz et al., 1993); (2) Gly80 (TM2), necessary for conformational transitions during the transport process (Zhou and Kanner, 2005); (3) Tyr140 (TM3), involved in substrate recognition and transport (Bismuth et al., 1997). One of the major novel findings recently emerged from the crystal structure of $LeuT_{Aa}$ is that there is an internal structural repeat that allows an ideal superimposition of TM1-5 with TM6-10 by a 176.5° rotation around a pseudo-two-fold axis located in the plane of the membrane (Yamashita et al., 2005). TM1 and TM6 are oriented antiparallel to each another, with breaks in their helical structure half-way through the cell membrane. In the substrate-bound, outward-occluded and competitive inhibitor-bound outward-facing conformation, Val23 and Gly24 in TM1 and the aminoacidic residues between Ser256 and Gly260 in TM6 have extended non-helical conformations, which expose atoms that can be used for hydrogen bonding and ion coordination. The regions surrounding these unwound breaks in TM1 and TM6, together with other regions in TM3 and TM8, comprise the substrate and Na^+-binding sites (Yamashita et al., 2005). Hinge movements allow TM1b, TM2a, TM6a to pivot around Val23, Gly55, and Leu257 and move outwards, in the Na^+-bound, substrate-unbound outward open conformation. These movements cause the extracellular loop 3 (EL3) and TM11 to be displaced by 2.8Å and 2.2Å, respectively (Krishnamurthy and Gouaux, 2012). In the inward-open conformation, TM1b and TM6a move and block the extracellular pathway, while TM1a is tilted by 45o with respect to its position in the closed state (Krishnamurthy and Gouaux, 2012). TM2, TM5, and TM7, domains that buttress TM1 and TM6, bend and cause EL4 to move down into the extracellular vestibule and close the extracellular solvent pathway. These structural rearrangements of $LeuT_{Aa}$ are consistent with an alternating access transport mechanism, whereby conformational changes in the structure of a transporter switch the accessibility of the substrate-binding site from the extracellular and cytoplasmic side of the membrane (Jardetzky, 1966; Lauger et al., 1980). The probability of a transporter of being in one state vs. the other depends on the energetic barriers associated with each conformational transition, which in turn depend on the substrate and co-transported ion concentration and on the membrane potential (Chung and Eaton, 2013; Schuler and Clarke, 2013).

THE DISTRIBUTION OF GABA TRANSPORTERS IN THE BRAIN

In situ hybridization studies have shown that the mRNA encoding GABA transporters is widely distributed throughout the entire central nervous system (Durkin et al., 1995; Borden, 1996). Intense labeling for the GABA transporter GAT1 is found in the cerebellum (molecular layer), basal ganglia (ventral pallidum, globus pallidus), olfactory bulb (glomerular layer), retina (inner nuclear layer), and interpeduncular nucleus. Moderate labeling is found throughout the neocortex (hippocampus proper and dentate gyrus), amygdala, septum, thalamus (ventral lateral geniculate, reticular nuclei), zona incerta, subthalamic nucleus, hypothalamus (suprachiasmatic and periventricular nuclei, anterior hypothalamic and pre-optic areas), superior colliculus, dorsal tegmental nuclei, basal ganglia (substantia nigra), nucleus of Darkschewitsch, pons and medulla (trapezoid, medial and lateral vestibular, dorsal cochlear and parabrachial nuclei, nucleus of the solitary tract and of the trigeminal nerve) and also in the spinal cord (dorsal horn laminae 1, 2, 4, 10). Weak labeling is found in cerebellar Purkinje cells, deep cerebellar nuclei and also in the spinal cord (ventral horn). In contrast to GAT1, the mRNA encoding the GABA transporter GAT2 is only detected in the leptomeninges, possibly suggesting a role for GAT2 in

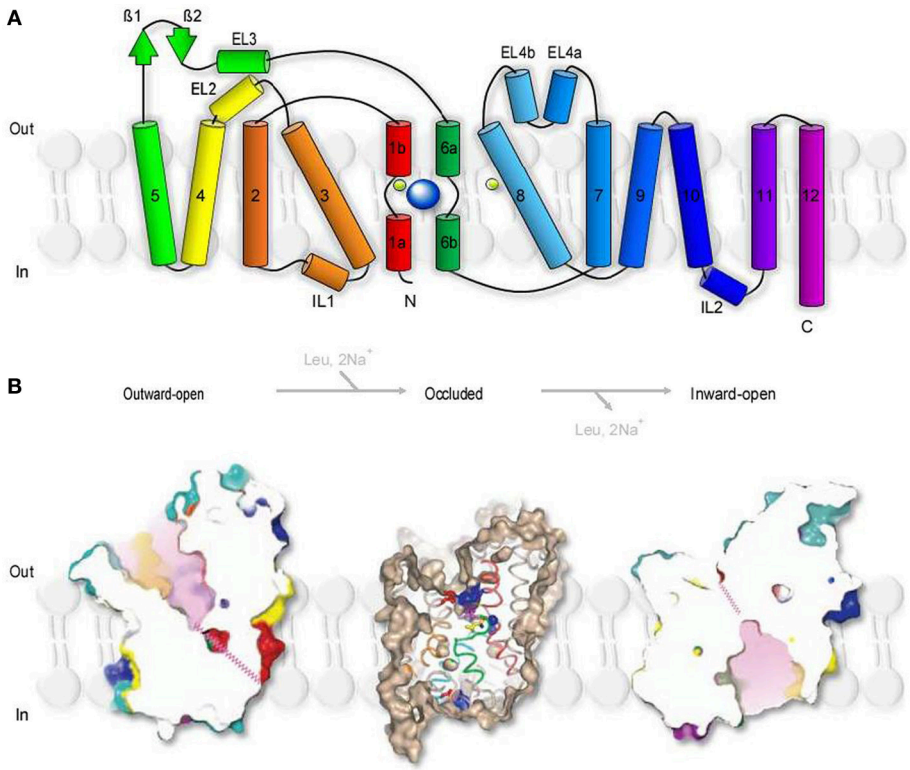

FIGURE 2 | Secondary structure and surface representation of $LeuT_{Aa}$. **(A)** Topology of *Aquifex aeolicus* $LeuT_{Aa}$. The transporter is composed of 12 trans-membrane regions (TM1-12), with cytoplasmic N- and C-terminal domains. TM1 and TM6 are oriented antiparallel to one another and have breaks in their helical structure approximately halfway across the membrane bilayer. The transporter has two extracellular β-strands (green arrows), four extracellular (EL2, 3, 4a, 4b) and two intracellular helices (IL1, 2). The co-transported Na^+ are depicted as two light green spheres. The substrate molecule (Leu), is depicted as a bigger blue sphere that binds to unwound regions in TM1 and TM6. Modified from (Yamashita et al., 2005). **(B)** Slice through the surface representation of $LeuT_{Aa}$ in the Leu-free, Na^+-bound outward-open conformation (left), in the occluded conformation where the Leu- and Na^+-binding sites are occluded from solution in the extracellular and cytoplasmic sides (middle) and in the inward-open conformation (right). The zig-zag pink lines indicate closed intracellular pathways. Modified from (Yamashita et al., 2005) and (Krishnamurthy and Gouaux, 2012).

regulating the GABA concentration or the osmotic pressure in the cerebrospinal fluid. The mRNA encoding the GABA transporter GAT3 is less abundantly expressed than the one for GAT1. Intense labeling is found in the olfactory bulb (glomerular layer) and retina (inner nuclear layer). Moderate labeling is found in the septum (medial nucleus and vertical nucleus of the diagonal band), basal ganglia (ventral pallidum and globus pallidus), subfornical organ, amydala, thalamus (paraventricular nucleus, lateral habenula), superior colliculus, ventral tegmental nucleus, basal ganglia (substantia nigra pars compacta), and medial vestibular nucleus. Weak labeling is found in lateral reticular and parabrachial nuclei, deep cerebellar nuclei, spinal cord and in the entire neocortex (Borden, 1996). The immunohistochemical analysis largely confirms these results, particularly the faint expression of GAT2 throughout the central nervous system, as opposed to other peripheral organs (Ikegaki et al., 1994), and the widespread distribution of GAT1 and GAT3 throughout the brain (Ikegaki et al., 1994). The highest levels of expression of GAT1 are found in the hippocampus, olfactory bulb, cortical layer 1 (L1), piriform cortex, superior colliculus, interpeduncular nucleus and nucleus spinal tract of the trigeminal nerve. The highest levels of expression of GAT3 are found in the olfactory bulb, thalamus, hypothalamus, pons and medulla, globus pallidus, basal ganglia (substantia nigra), deep cerebellar nuclei, and nucleus spinal tract of the trigeminal nerve (Ikegaki et al., 1994). The density of expression of GAT1-3 in the cortex varies across layers (**Figure 3**). GAT1 immunostaining is highest in L2-L4, GAT2 is confined to the meninges and GAT3 is most highly expressed in L3 and upper L5 (Minelli et al., 1996; Conti et al., 2004).

THE CELLULAR AND SUB-CELLULAR DISTRIBUTION OF GABA TRANSPORTERS

One of the most interesting features of GAT1 and GAT3 is their cellular and sub-cellular distribution.

In rodents, the punctate immunostaining for GAT3 is localized exclusively in astrocytic processes scattered throughout the neuropil and adjacent to symmetric and asymmetric synapses close to cell bodies, basal and apical dendrites (Minelli et al., 1996; Ribak et al., 1996; Melone et al., 2005 but see Pow et al., 2005). In the brain of other mammalian species, like cats, monkeys and humans, the astrocyte-specific expression of GAT3 is lost, because here GAT3 is also expressed in oligodendrocytes (Pow et al., 2005).

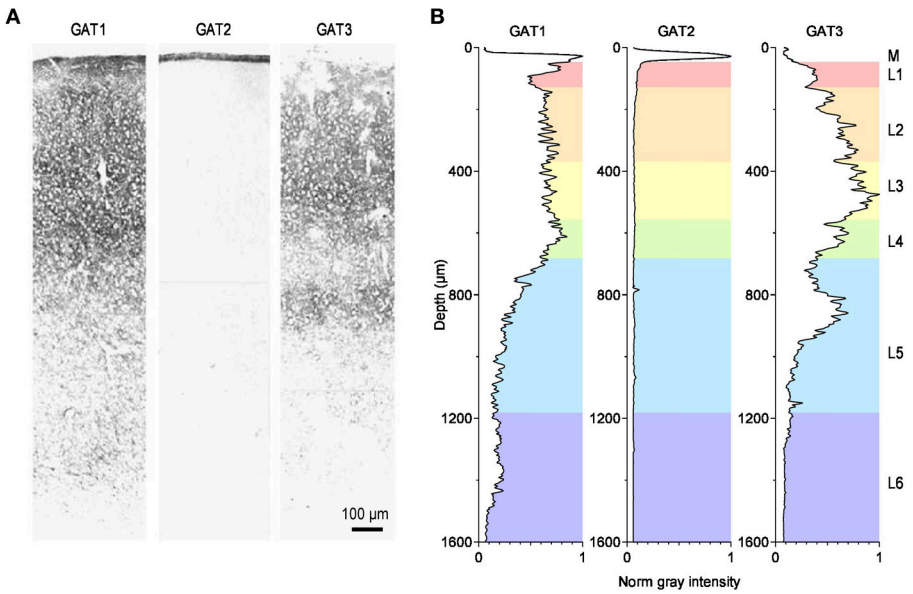

FIGURE 3 | Distribution of GABA transporters in the rat somato-sensory cortex. (A) Immunohistochemical labeling for the GABA transporters GAT1 (left), GAT2 (middle) and GAT3 (right) in the primary somato-sensory cortex of the adult rat. Modified from (Conti et al., 2004). **(B)** Image analysis of the immunohistochemical labeling for GAT1-3. The diagrams provide a measure of the normalized, average gray value distribution measured over the entire area of the images shown in panel A. The data are normalized by the maximum gray value measured in each image. Therefore, the darkest areas, with the most intense labeling, have a normalized gray intensity value of 1. The letters on the right hand side of the figure indicate the meningeal (M) and the six cortical layers (L1–6). The gray value analysis was done using the Fiji image processing package (Schindelin et al., 2012). The rest of the analysis was performed using custom-made routines written in Igor Pro (Wavemetrics).

At the electron microscope level, the punctate GAT1 immunoreactivity is mainly confined to the axon terminals of symmetrical synapses in the neocortex (**Figure 4A**). For the most part, this labeling overlaps with that of GAD67-positive terminals located near neuronal cell bodies, axon initial segments and proximal dendrites (Minelli et al., 1995; Ribak et al., 1996), but some GAT1 immunoreactivity has also been detected in distal astrocytic processes (Minelli et al., 1995; Ribak et al., 1996) and post-synaptically, in the dendrites and soma of non GABAergic neurons (Yan et al., 1997). The pre-synaptic neuronal location of GAT1 seems suited for GABA recycling in the pre-synaptic terminal following a release event. This suggests that GABA uptake, not only GABA biosynthesis, is essential to sustain GABAergic synaptic transmission. The function of post-synaptic GAT1 in incompletely understood, but if we could draw an analogy with what we have learnt from the activity of neuronal post-synaptic transporters at excitatory synapses (Scimemi et al., 2009), we would probably infer that they may limit GABA escape from the synaptic cleft toward extra-synaptic territories. The density of expression of GAT1 in cortical and cerebellar plasma membranes has been estimated with fluorescent labeling experiments (Chiu et al., 2002). According to these findings, GAT1 is expressed at a density of 500–800 μm^{-2}, 61–63% of the total density of expression of GAT1 in intracellular and plasma membranes (800–1300 μm^{-2}). These tentative estimates are useful to develop quantitative frameworks to determine the function of GABA transporters in the synapse (Scimemi, 2014). Because they represent average *density* values, however, they imply that synapses with a large surface area express a higher *number* of GABA transporters than small synapses. There is no clear experimental evidence that supports this assumption. Another important piece of information that is currently missing is whether the relative abundance of GAT1 vs. GAT3, and their relative contribution to synaptic function, varies across synapses with different levels of astrocytic coverage. Therefore, one of the current major limitations is that our understanding of GABA transporters is based on "average" quantitative estimates that do not capture the effect of cell-to-cell variability in GABA transporter expression/activity on synaptic function. There are in fact indications that the expression level of GAT1 vary significantly across GABAergic interneurons. For example, in the hippocampus, GAT1 is more abundantly expressed in *stratum radiatum* rather than *stratum oriens* interneurons (Engel et al., 1998). Accordingly, the amplitude and time course of GABAergic IPSCs evoked in CA1 pyramidal cells by stimulating *stratum radiatum* interneurons show higher sensitivity to the GAT1 inhibitor tiagabine than those evoked by stimulating *stratum oriens* interneurons (Engel et al., 1998). There are examples of GABAergic cells, like cerebellar Purkinje neurons, that lack GAT1 and any designated mechanism for GABA uptake in the pre-synaptic terminal (Minelli et al., 1995). Here the GABA transporter responsible for GABA clearance from the extracellular space is GAT3, which is highly expressed in Bergmann glial cell processes surrounding the synaptic terminal (**Figure 4B**). Something similar occurs in the thalamus, where GABA transporters are only expressed in astrocytes (De Biasi et al., 1998; Vitellaro-Zuccarello et al., 2003). Interestingly, in the thalamus,

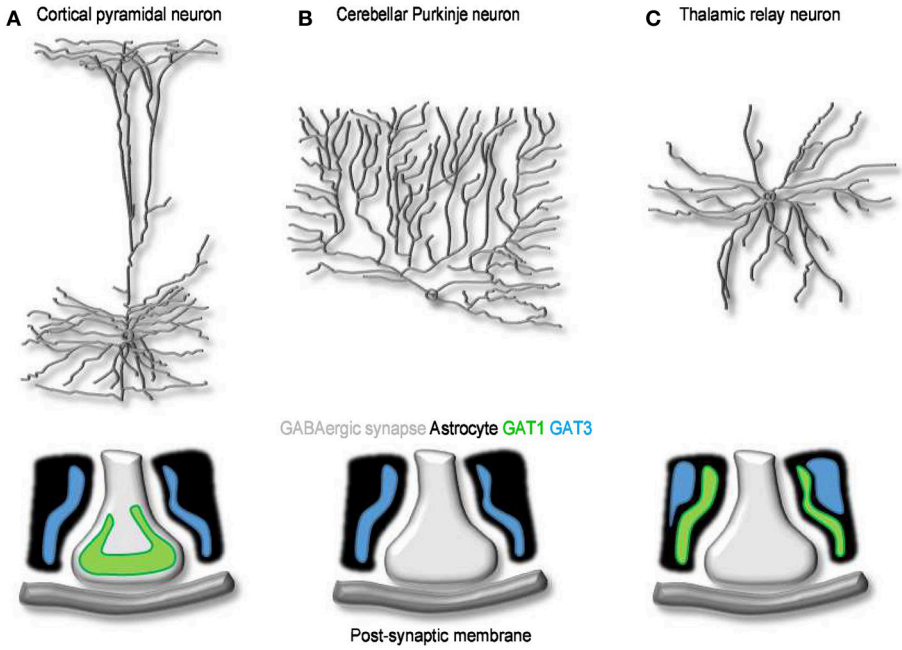

FIGURE 4 | The cellular and sub-cellular distribution of GABA transporters. (A) Schematized morphology of cortical pyramidal neuron (top) and of the distribution of GAT1 (green) and GAT3 (blue) at synaptic contacts onto these cells (bottom). **(B,C)** As in A, for Purkinje **(B)** and thalamic relay neurons **(C)**. At GABAergic synapses onto cortical neurons, GAT1 and GAT3 are expressed mainly in pre-synaptic terminals and in neighboring astrocytic processes, respectively. Purkinje neurons lack neuronal GABA transporters; Bergmann glia cells express GAT3. GABA uptake at synaptic contacts onto thalamic relay neurons is mediated by GAT1 and GAT3. Both transporters are located in astrocytes: GAT1 is located closer to the synapse and clears GABA released during phasic events; GAT3 is located further away and regulates the basal, tonic GABA concentration in the extracellular space (Beenhakker and Huguenard, 2010).

GAT1 and GAT3 may occupy distinct domains within the astrocytic membrane, with GAT1 located closer to synaptic contacts than GAT3 (Beenhakker and Huguenard, 2010) (**Figure 4C**). This peculiar distribution may allow GAT1 and GAT3 to have different roles on synaptic function: the former limiting GABA escape from the synaptic cleft during phasic synaptic transmission, the latter controlling the ambient GABA concentration mediating tonic inhibition (Beenhakker and Huguenard, 2010). These findings are consistent with the recently emerging view that GAT1 and GAT3 regulate different signaling pathways, mediated by GABA released via vesicular and non-vesicular mechanisms (Song et al., 2013), during low-frequency or sustained neuronal activity (Kersante et al., 2013).

THE FUNCTIONAL ROLE OF GABA TRANSPORTERS

GABA transporters can transiently bind extracellular GABA, remove it from the extracellular space and, under appropriate ionic conditions, translocate it from the cytosplasm back into the extracellular space (**Figure 5A**). The diversity of these effects indicates that the functional implications of GABA uptake on synaptic transmission are multiple and complex. There have been apparently conflicting results on the ability of GABA transporters to control the time course of GABAergic currents. This is because inhibiting GABA uptake markedly prolongs the neuronal response to iontophoretic GABA applications (Curtis et al., 1976; Brown and Galvan, 1977; Brown et al., 1980; Alger and Nicoll, 1982; Dingledine and Korn, 1985) and to repetitive synaptic stimulations (Roepstorff and Lambert, 1992; Thompson and Gahwiler, 1992; Isaacson et al., 1993; Draguhn and Heinemann, 1996; Overstreet and Westbrook, 2003) (**Figure 5B**). It also prolongs the late phase of evoked inhibitory post-synaptic currents [IPSCs; (Dingledine and Korn, 1985; Isaacson et al., 1993)] but has little effect on the amplitude and initial decay of action potential independent miniature and unitary IPSCs (Dingledine and Korn, 1985; Rekling et al., 1990; Thompson and Gahwiler, 1992; Isaacson et al., 1993; Rossi and Hamann, 1998; Overstreet and Westbrook, 2003) (**Figure 5B**). The discrepancy between these results has been partly resolved over the years based on theoretical and experimental works showing that: (1) the ability of GABA transporters to alter receptor activation depends on the amplitude and time course of the GABA concentration transient; (2) GABA diffusion and GABA receptor kinetics—not GABA uptake—shape the profile of synaptic currents evoked by brief agonist concentrations; (3) GABA uptake regulates GABA receptor activation during simultaneous recruitment of neighboring synapses; (4) GABA uptake limits GABA escape from active synapses (i.e., spillover) and therefore controls the spatial specificity of GABAergic transmission (Dingledine and Korn, 1985; Isaacson et al., 1993; Overstreet et al., 2000, 2003). When GABA uptake is intact, there is a progressive decline in the proportion of time that $GABA_A$ receptors spend in the open vs. desensitized state at increasing distances from active release

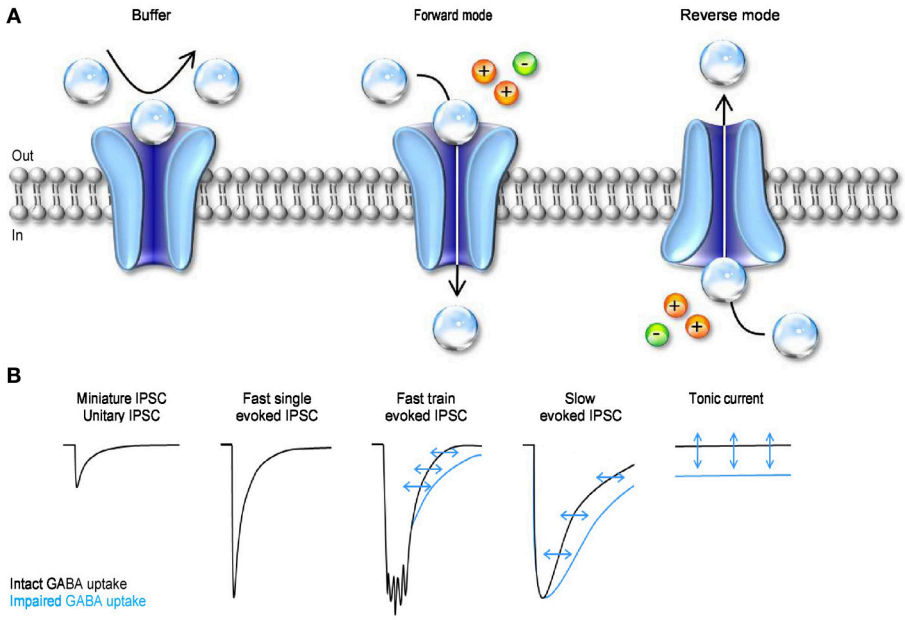

FIGURE 5 | The mode of action and functional effect of GABA transporters on synaptic transmission. (A) Schematic representation of the three different modes of action of GABA transporters. GABA molecules (transparent spheres) can be rapidly bound by GABA transporters. Not all the GABA molecules that are bound by the transporters are also translocated across the cell membrane. Under these conditions, the transporters act as buffers (left). GABA uptake is coupled to the movement of Na^+ (orange sphere) and Cl^- (green sphere) across the membrane. In forward mode, GABA transporters remove GABA from the extracellular space (middle). GABA transporters can operate in revered mode (i.e. release GABA in the extracellular space) if the driving force for Na^+/Cl^- favors the movement of these ions outside the cell, and if the intracellular concentration of GABA is sufficiently high to be bound by the transporters (right). **(B)** Schematic representation of the effects of GABA uptake on small, fast, coincident and tonic GABAergic currents. The black traces represent currents recorded in control conditions, with GABA uptake intact. The blue traces represent currents recorded when GABA transporters are blocked.

sites (Overstreet et al., 2003). This decline becomes more gradual when GABA transporters are blocked, because a larger fraction of $GABA_A$ receptors away from the release site open, rather than desensitize, following GABA release (Overstreet et al., 2003). Taken together, these findings indicate that a key role of GABA transporters in cortical microcircuits is to convert a spatially confined signal into a spatially unrestricted wave of inhibition capable of activating a broad range of pre- and post-synaptic $GABA_A$ and $GABA_B$ receptors. The likelihood with which these broad range effects occur varies across synapses, depending on the agonist concentration profile and the diffusion properties of the synaptic and peri-synaptic environments. In the hippocampus, GABA release from parvalbumin-expressing (PV) interneurons evokes post-synaptic currents that last <1 ms. In contrast, the currents evoked by GABA release from neurogliaform cells last >100 ms (Hajos et al., 2000; Tamas et al., 2003; Olah et al., 2009; Barberis et al., 2011; Capogna and Pearce, 2011; Chittajallu et al., 2013). Slow GABAergic currents are more susceptible to the activity of GABA transporters, suggesting that slow waves of inhibition, rather than rapid point-to-point communication is mainly modulated by GABA transporters (Szabadics et al., 2007). In hippocampal CA1 pyramidal cells, blocking GABA uptake causes a more pronounced increase in somatic than dendritic currents evoked by exogenous GABA applications (Isaacson et al., 1993). This effect has been attributed to the existence a larger proportion of somatic, rather than dendritic, GABAergic inputs onto these cells. As a consequence, the local concentration of GAT1 (the most abundant GABA transporter in this brain region) is higher at the soma than along the dendrites of CA1 pyramidal cells (Gulyas and Freund, 1996; Miles et al., 1996). Interestingly, many peri-somatic GABAergic inputs onto CA1 pyramidal cells come from PV interneurons (Freund and Buzsaki, 1996; Klausberger and Somogyi, 2008). It is therefore puzzling that there is a high concentration of GAT1 in a sub-cellular compartment where individual GABAergic IPSCs have rapid kinetics and are largely unaffected by blocking GABA uptake. One possible explanation is that peri-somatic GAT1 serves to limit GABA diffusion away from the somatic region during repetitive activation of PV cells and toward the somatic region following the onset of slow waves of dendritic inhibition. If confirmed, this hypothesis may suggest that the main functional role of peri-somatic GABA transporters is to maintain a spatial separation between somatic and dendritic GABAergic signals.

An additional form of GABAergic signaling is the one mediated by tonic activation of GABA receptors (Brickley et al., 1996). These currents are due to the continuous presence of a low submicromolar concentration of GABA in the extracellular space (Scimemi et al., 2005; Santhakumar et al., 2006; Wu et al., 2007). That is to say that GABA transporters, despite being able to remove GABA from the extracellular space, do not lower its

concentration to levels that prevent it from activating GABA receptors. Tonic GABA currents have been recorded in a variety of cells *in vitro* (Brickley et al., 1996; Rossi and Hamann, 1998; Hamann et al., 2002; Nusser and Mody, 2002; Mitchell and Silver, 2003; Semyanov et al., 2003; Stell et al., 2003; Caraiscos et al., 2004; Scimemi et al., 2005; Wojtowicz et al., 2013) and *in vivo* (Chadderton et al., 2004; Duguid et al., 2012; Kersante et al., 2013; Rovo et al., 2014) (**Figure 5B**). The functional role of tonic GABA currents is to reduce the neuronal input conductance (Cavelier et al., 2005; Farrant and Nusser, 2005), but the consequences that this has on cell excitability depend on the temporal profile of incoming excitatory inputs (Mitchell and Silver, 2003). The tonic GABA current offsets the cell's output firing rate in response to sustained depolarization and alters the slope of the cell's input-output relationship in response to a random train of excitatory inputs (Mitchell and Silver, 2003). In the cerebellum, it enhances the sensitivity of granule cells to evoked vs. spontaneous sensory stimulation (Duguid et al., 2012) and in the thalamus it controls the onset of neural network oscillations (Rovo et al., 2014). Therefore, by regulating tonic forms of signaling, GABA transporters can exert a powerful control of the accuracy with which information is relayed across cells and brain regions. The electrochemical gradient for Na^+/Cl^- determines the direction of GABA transport, and therefore the ability of GABA transporters to reduce or increase the extracellular GABA concentration. Accordingly, previous reports indicate that GABA transporters can operate in reverse mode (Wu et al., 2007). They do so under experimental conditions that mimic ischemia (Allen et al., 2004), but there is an ongoing debate on whether reverse GABA uptake typically occurs under more physiological conditions (Heja et al., 2012; Wojtowicz et al., 2013).

THE REGULATION OF GABA TRANSPORTER DENSITY OF EXPRESSION

GABA transporters, like other neurotransmitter transporters, are dynamically regulated at the level of their surface-to-cytoplasm partitioning and anchoring to the cell membrane (**Figure 6**). In *Xenopus* oocytes, activating protein kinase C (PKC) with phorbol esters like phorbol-12-myristate-13-acetate (PMA) and 1-oleoyl-2-acetyl-sn-glycerol (OAG), or with *N*-heptyl-5-chloro-1-naphtalenesulphonamide (SC-10) and (-)-indolactam V (indoV) enhances GABA uptake via GAT1, whereas inhibiting PKC with bisindolyilmaleimide reduces it (Corey et al., 1994). Consistent with these findings, inhibiting the phosphatase PP2B (calcineurin) enhances GABA uptake (Corey et al., 1994). Notably, the effects of PKC and PP2B on GABA uptake are entirely post-translational because they can be detected even in the presence of cycloheximide, a protein synthesis inhibitor. PKC and PP2B are thought to alter the trafficking of GAT1 from trans-Golgi and/or low density cytoplasmic vesicles to the plasma membrane by modulating the phosphorylation state of non-consensus sites on GAT1 and/or the activity of second messenger signaling cascades. These changes in the surface-to-cytoplasm expression of GAT1 are associated with changes in the translocation rate of GABA via GAT1 (V_{max}), not in the Michaelis-Menten constant for GABA uptake via GAT1 (K_m), suggesting that they do not alter GAT1 binding affinity for the substrate (Corey et al., 1994). The magnitude of these effects depends on the abundance of expression of GAT1 in the plasma membrane: the more GAT1 is present on the plasma membrane, the smaller is the modulatory effect of PKC. However, any attempt to generalize the findings obtained in *Xenopus* oocytes to other cell types should be pursued with caution. In rat brain cortical cultures, pharmacological activating PKC with PMA, phospholipase C (PLC) or OAG (the endogenous, membrane permeable analog of DAG generated from phosphatidylinositol breakdown by PLC) does not alter GABA uptake in neurons but decreases it in glial cells (Gomeza et al., 1991). In rat hippocampal cultures, PMA reduces GABA uptake in neurons but not in glial cells (Beckman et al., 1998). Enhancing PKC activity indirectly, with agonists of G-protein-coupled neurotransmitter receptors that lead to PKC activation also reduces GABA uptake in neurons (Beckman et al., 1999). Accordingly, agonists of acetylcholine muscarinic receptors (M1, M3 and M5), glutamate metabotropic receptors (group I) and serotonin receptors (5-HT2) all reduce GABA transporter cell membrane expression by activating PKC (Beckman et al., 1999). Although puzzling and largely unresolved, the discrepancy in the effect of PKC activation on GABA transporters in *Xenopus* oocytes, cultured neurons and glial cells may be attributed to differences in the concentration of PKC in all these different cell types. This suggests that *in vivo* the influence of PKC on GAT1 activity may vary significantly depending on the amount and specific PKC isoform expressed in different neurons and, possibly, in different sub-cellular compartments (Takai et al., 1977; Nishizuka, 1988).

A distinct mechanism through which neurons can regulate the cell-surface expression of GAT1 is via tyrosine kinase phosphorylation of intracellular tyrosine residues (Law et al., 2000) (**Figure 6A**). These changes in the cell-to-cytoplasm partitioning of GAT1 are due to reduced internalization of the transporter during tyrosine kinase activation (Whitworth and Quick, 2001). In rat hippocampal neurons, the effect of tyrosine kinases activation is similar to that evoked by PKC inhibition, namely an increase in GABA uptake due to enhanced cell membrane expression of GAT1. Under physiological conditions, a possible candidate trigger for tyrosine kinases activation is the growth factor BDNF, which is synthesized and secreted by pyramidal neurons but has target receptors in various types of GABAergic interneurons (Ernfors et al., 1990). During development, GABA-induced membrane depolarization leads to opening of L-type voltage-gated calcium channels and release of BDNF, which in turn promotes interneuron differentiation (Marty et al., 1996a,b). By increasing GABA uptake, BDNF may alter the concentration of GABA in pre-synaptic terminals and its lifetime in the extracellular space. As GABA becomes hyperpolarizing (Ben-Ari et al., 1989), it loses its ability to activate L-type calcium channels and trigger BDNF synthesis and release (Berninger et al., 1995). The consequent reduction in GABA uptake may contribute to the reduced neurotrophic effects of GABA in the mature brain.

THE REGULATION OF GABA TRANSPORTER LATERAL MOBILITY

Previous works indicate that the localization of individual GAT1 molecules within the plasma membrane is not fixed in time and

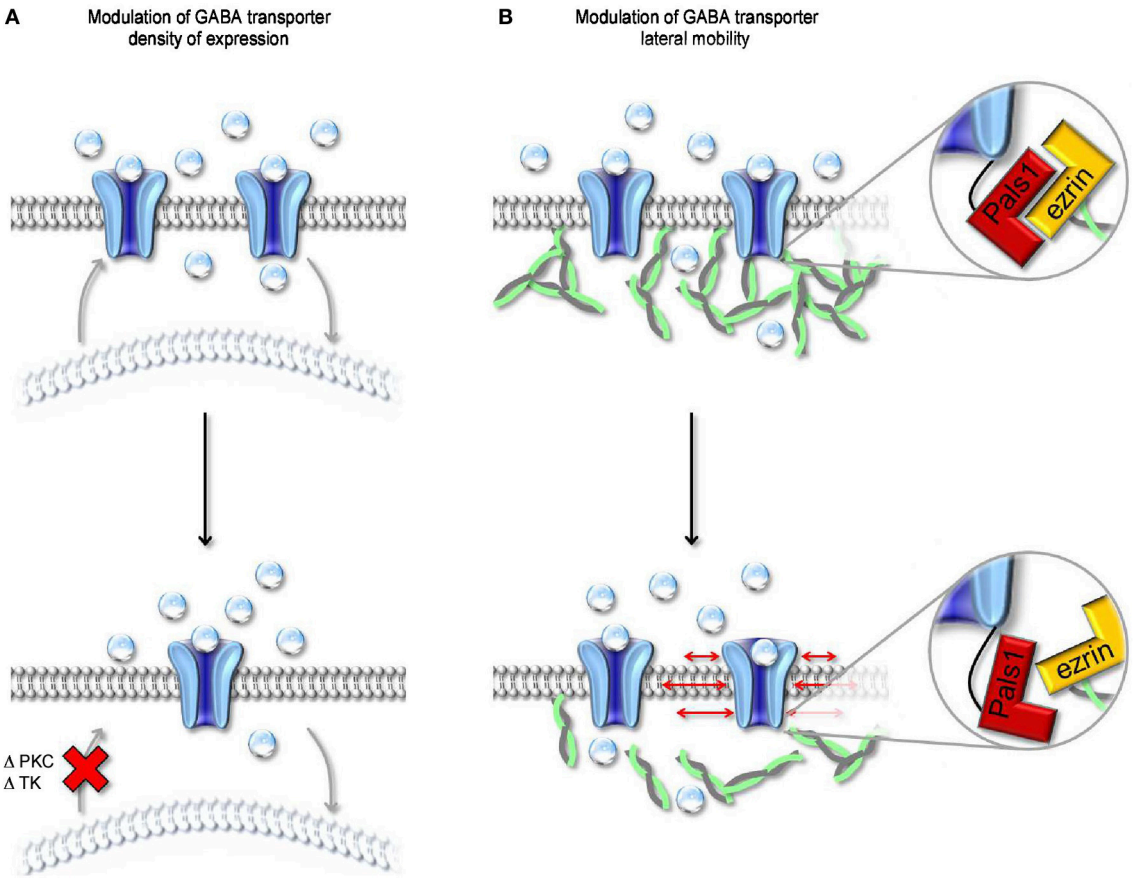

FIGURE 6 | Modulation of surface expression and lateral mobility of GABA transporters. (A) Schematic representation of changes in cell surface expression of GABA transporters evoked by altering the activity of PKC and/or tyrosine kinase (TK). Both kinases regulate the trafficking of GABA transporters from intracellular organelles (light, curved lipid bilayer) to the cell membrane (dark, flat lipid bilayer). A reduction in the rate of cell membrane insertion (bottom) leads to a decrease in the cell surface expression of GABA transporters. **(B)** Schematic representation of the molecular interactions mediating anchoring of GABA transporters to the actin cytoskeleton. The MAGUK protein Pals1 mediates the interaction between the C-terminal of GABA transporters and ezrin, an adaptor protein that interacts with actin. Disruption of the interaction between Pals1 and ezrin leads to an increased lateral mobility of GABA transporters in the cell membrane.

is not entirely predicted by passive diffusion in the lipid bilayer (Imoukhuede et al., 2009). This is in agreement with the results of FRAP experiments in neuroblastoma 2a cells, in which 50% of the total pool of GAT1 present in the cell membrane is mobile while the remaining 50% is immobile. Within the immobile population, a fraction of GAT1 molecules is indirectly tethered to the actin cytoskeleton via a PDZ-interaction between the C-terminal domain of GAT1, the MAGUK protein Pals1 (Mchugh et al., 2004) and ezrin, an adaptor protein that connects Pals1 to actin (Imoukhuede et al., 2009) (**Figure 6B**). Disrupting the interaction between GAT1 and the actin cytoskeleton (i.e., rendering all GAT1 molecules mobile) has been shown to increase GABA uptake (Imoukhuede et al., 2009). However, these conclusions are based on experimental measures of radioactive tracer flux typically obtained under steady-state conditions (i.e., in the presence of an unknown, but constant agonist concentration). There is hardly anything that is at steady-state during synaptic transmission. For example, the GABA concentration varies dramatically with time and distance from the release site. It is therefore challenging to get a simple and accurate intuition of whether and how changes in the lateral mobility of GABA transporters can affect the GABA concentration profile inside and outside the synaptic cleft. A simple modeling approach suggests that increasing the mobility of GABA transporters along the cell membrane does not alter the lifetime of GABA in the extracellular space but allows it to diffuse further away from an active release site (Scimemi, 2014). This suggests that intracellular signaling cascades that regulate the surface expression and mobility of GABA transporters may ultimately control the spatial specificity of GABAergic transmission in the brain.

CONCLUSIONS

There have been tremendous advances in our understanding of the molecular architecture and function of GABA transporters. A number of works has shown that there are intracellular signaling cascades that control the cell surface expression and mobility of GABA transporters, suggesting that the regulation of GABA transporter activity may be more complex than

previously thought. It remains currently unclear how these short- and long-term changes in the expression, mobility and activity of GABA transporters affect the time course and spatial spread of GABAergic signals in the brain. This missing piece of information hold the promise of providing novel insights into the regulation of synaptic transmission by GABA transporters and will shed new light on our current understanding of the synaptic mechanisms underlying the generation and maintenance of neuronal network oscillations in distinct regions of the living brain.

ACKNOWLEDGMENT

Thanks to E. Gouaux and H. Owen for their support with the preparation of **Figure 2** and P. E. Forni and A. M. Sinensky for valuable comments on the manuscript.

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

Received: 23 April 2014; accepted: 27 May 2014; published online: 17 June 2014.
Citation: Scimemi A (2014) Structure, function, and plasticity of GABA transporters. Front. Cell. Neurosci. ***8****:161. doi: 10.3389/fncel.2014.00161*
This article was submitted to the journal Frontiers in Cellular Neuroscience.

frontiers in
CELLULAR NEUROSCIENCE

ORIGINAL RESEARCH ARTICLE
published: 10 June 2014
doi: 10.3389/fncel.2014.00151

Plasticity of $GABA_A$ receptor diffusion dynamics at the axon initial segment

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The axon initial segment (AIS), a site of action potential initiation, undergoes activity-dependent homeostatic repositioning to fine-tune neuronal activity. However, little is known about the behavior of $GABA_A$ receptors ($GABA_A$Rs) at synapses made onto the axon and especially the AIS. Here, we study the clustering and lateral diffusion of $GABA_A$Rs in the AIS under baseline conditions, and find that $GABA_A$R lateral mobility is lower in the AIS than dendrites. We find differences in axonal clustering and lateral mobility between $GABA_A$Rs containing the α1 or α2 subunits, which are known to localize differentially to the AIS. Interestingly, we find that chronic activity driving AIS repositioning does not alter GABAergic synapse location along the axon, but decreases $GABA_A$R cluster size at the AIS. Moreover, in response to chronic depolarization, $GABA_A$R diffusion is strikingly increased in the AIS, and not in dendrites, and this is coupled with a decrease in synaptic residency time of $GABA_A$Rs at the AIS. We also demonstrate that activation of L-type voltage-gated calcium channels is important for regulating $GABA_A$R lateral mobility at the AIS during chronic depolarization. Modulation of $GABA_A$R diffusion dynamics at the AIS in response to prolonged activity may be a novel mechanism for regulating GABAergic control of information processing.

Keywords: gaba receptors, homeostatic plasticity, axon initial segment, calcium, quantum dots, diffusion

INTRODUCTION

The axon initial segment (AIS), a neuronal subdomain enriched with ion channels, scaffolding components and cytoskeletal elements, serves as a key site for action potential initiation, and separates neuronal input and output domains (Rasband, 2010). Several proteins, including Na^+ channels, the scaffolds ankyrin-G (ankG) and βIV-spectrin, and the cellular adhesion molecule neurofascin 186, form a protein-dense segment of approximately 20 μm in length, located near to the cell soma (Rasband, 2010). The AIS can also translocate away from the cell soma in response to altered neuronal activity patterns (elevated extracellular K^+, Grubb and Burrone, 2010), with all AIS proteins tested (including ankG, NaV channels and NF 186) found to undergo a distal shift of approximately 10 μm along the axon. This structural plasticity, which depends on activation of voltage-gated calcium channels, results homeostatically in increased thresholds for action potential firing (Grubb and Burrone, 2010; O'Leary et al., 2010).

The AIS also receives GABAergic input from axo-axonic interneurons, which contact AIS-localized postsynapses containing clusters of $GABA_A$Rs, while other neurotransmitter receptors are primarily absent from the AIS (Kole and Stuart, 2012). $GABA_A$Rs are the major mediators of fast synaptic inhibition in the brain, though evidence suggests that axo-axonic inputs can also be depolarizing or excitatory (Szabadics et al., 2006; Khirug et al., 2008; Kole and Stuart, 2012), thus possibly providing a dual function. It is clear that synapses made onto the AIS can control cell excitability, firing frequency and input–output relationship (Klausberger and Somogyi, 2008; Kole and Stuart, 2012). $GABA_A$Rs containing α1, α2, or α3 subunits are found enriched at synapses while α4, α5, and α6 are found primarily extrasynaptically (Luscher et al., 2011). Of the synaptic α subunits, α2 subunits (and α3 in some cell types) are enriched at the AIS, while few $GABA_A$Rs at the AIS contain the α1 subunit (Nusser et al., 1996; Brünig et al., 2001; Panzanelli et al., 2011). While $GABA_A$R membrane dynamics have been well studied in dendrites, including their lateral diffusion into and out of synapses (Thomas et al., 2005; Bannai et al., 2009; Muir et al., 2010), virtually nothing is known about the clustering and lateral mobility of $GABA_A$Rs at the AIS. Moreover, whether GABAergic AIS synapses shift away from the soma in response to chronic depolarization, or whether the diffusion dynamics of $GABA_A$Rs at the AIS can be modified is unknown.

Here, we investigate the subunit-specific differences between α1- and α2- containing $GABA_A$Rs in terms of their clustering and lateral mobility at the AIS. We find that α2 clusters are more numerous in the axon than α1 clusters, and that $GABA_A$R lateral mobility at the AIS is lower for α2- vs. α1-containing $GABA_A$Rs. While the AIS moves away from the cell body in response to chronic depolarization, GABAergic pre- and postsynaptic elements remain fixed in position along the axon. In contrast, $GABA_A$R lateral mobility in the AIS and proximal axon is specifically increased in response to chronic depolarization, coupled with decreased residency time at AIS synapses and reduced $GABA_A$R cluster size in the AIS. Increased AIS-$GABA_A$R lateral mobility is caused by activation of L-type VGCCs, which also drives AIS translocation (Grubb and Burrone, 2010). Our results provide a novel mechanism for modulation of GABAergic

synapses under conditions of prolonged activity, which could have important implications for control of neuronal activity and information processing.

MATERIALS AND METHODS

CELL CULTURE AND DRUG TREATMENTS

We used standard culture of primary dissociated hippocampal neurons from E18 embryonic rats as described previously (Banker and Goslin, 1991). For chronic depolarization, the extracellular potassium concentration was elevated from 5 to 15 mM by adding KCl from a 1M stock solution. Nifedipine was from Tocris. KCl (15 mM) and nifedipine (5 μM) treatments were made at 12 DIV for 48 h, and all experiments were performed at 14 DIV. Transfection of ankG-GFP (a kind gift from V. Bennett, HHMI) was made by calcium phosphate precipitation at 10 DIV as previously described (Twelvetrees et al., 2010). Transfection of mGFP was made by lipofectamine 2000 (Invitrogen) at 11 DIV, with 72 h expression before staining.

LIVE-CELL IMAGING

Imaging media used for quantum dot tracking experiments (Muir et al., 2010) contained 125 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM D-glucose, 10 mM HEPES and was adjusted to pH 7.4 with NaOH before use. Cells were imaged under perfusion (4 ml/min) and heating (35–37°C). Fluorescence was captured using an Olympus microscope (BX51WI) with a 60x Olympus objective coupled to an EM-CCD camera (Ixon, Andor). Excitation was provided by a mercury spiked xenon arc lamp (Cairn). Appropriate filters were chosen for QDs, alexa dyes, and FM 4–64.

Live labeling of the AIS (Schafer et al., 2009) was performed by mixing 1 μl of anti- pan-neurofascin (pan-NF, neuromab) with 0.35 μl of anti-mouse alexa 488 (Invitrogen). This mixture was incubated on ice for 15 min to allow coupling. Then, 100 μl block solution (imaging media containing 10% horse serum) was added and the solution kept at room temperature (RT) for 2–3 min. For parallel QD labeling of $GABA_A$Rs, rabbit anti- α1 or α2 (1:100, Synaptic Systems, both recognizing extracellular epitopes) was added to the pan-NF/alexa solution. Coverslips were incubated for 8 min at RT by inverting onto this solution spotted on film. Quantum dots (anti-rabbit 605 nm QD, 0.5 nM, Invitrogen) were attached with a subsequent 2 min labeling step in block solution, as above. Coverslips were washed 6–8 times in imaging media after each step. QD movies were of 200 frames, acquired at 8.5 Hz (movie length = 23.5 s). To minimize the amount of $GABA_A$R internalization within the recording period, movies were recorded within 15 min of QD labeling. Labeling of active presynaptic terminals with FM 4–64 (Invitrogen) was performed by 1 min incubations in 1 ml imaging media, first with 1 μm FM 4–64 + 60 mM KCl, followed by 0.2 μm FM 4–64. Coverslips were then washed extensively before imaging.

FIXED-CELL IMAGING

Co-staining for ankG and GABAergic synapse components (α1 and α2-$GABA_A$Rs, gephyrin, VGAT, all primary antibodies from Synaptic Systems, except ankG, neuroMab and γ2, a kind gift from J. M. Fritschy) was performed using standard immunofluorescence techniques. All primary antibodies were used at 1:100 with secondary staining at 1:500 with alexa 488/594 or cy5. Surface staining of $GABA_A$Rs (α1 or α2, both extracellular epitope) was made with an initial step in block solution lacking detergent. For analysis of AIS and cluster position, approximately 30 neurons were analysed per condition from images of the whole cell including 100 μm of axon (zoom = 0.7). For analysis of $GABA_A$R cluster size, images at 4 × zoom (25 μm length) were taken of the AIS and two regions of proximal dendrite chosen at random for each cell, and approximately 15 neurons per condition were imaged. All settings were kept constant across experiments. Confocal imaging was performed with Zeiss Pascal and Zeiss 700 microscopes equipped with 63× plan Apochromat oil objectives (NA 1.4).

IMAGE ANALYSIS

AIS position was measured from ankG staining using an automated detection routine similar to that used in (Grubb and Burrone, 2010). Briefly, the axon was traced in ImageJ (NIH) and straightened using the "Straighten" plugin. A running average of ankG intensity along the axon was made (window, $W = 20$ pixels approximately 5 μm). This image was then scaled such that its pixel intensities range from 0 to 1. Starting from the soma edge ($x = 0$), AIS start and end positions are defined as where the scaled ankG intensity first exceeds 0.33 and then drops below 0.33, respectively. To account for the size of the smoothing window, $W/2$ (approximately 2.5 μm) was added to output values of AIS start and end position. Good agreement was found between AIS start and length measurements as determined by this routine compared to analysis by manual inspection.

For analysis of the position of GABAergic synapse components along axons, the straightened image of the axon (as above) was used. Manual logging of each cluster position along the axon (up to 100 μm from the cell soma) was performed in ImageJ. Clusters were defined to be on the axon if their position overlapped with ankG staining and were classified as being before or within the AIS by manual inspection of cluster and AIS position (from ankG staining). $GABA_A$R cluster size was analysed using the "Analyse Particles" function in ImageJ. AIS and dendrite images were first intensity-thresholded (constant across experiments). AIS clusters were classified as those overlapping with AIS/ankG staining.

Analysis of QD-$GABA_A$R trajectories was performed as previously described (Muir et al., 2010) using custom detection and tracking software written in *Mathematica* (Wolfram Research). Instantaneous diffusion coefficients were calculated from the squared displacement across sequential trajectory segments of five frames, using the 2D diffusion relation, $<x^2> = 4Dt$. Diffusion coefficients were then pooled within like groups/conditions. To analyse QD-$GABA_A$R dynamics in different neuronal regions (i.e., AIS, proximal axon or sample dendrites), neuronal regions were first identified according to neurofascin staining and morphology and then isolated in ImageJ using the selection brush tool. For analysis of $GABA_A$R diffusion in synapses, QD-$GABA_A$Rs were defined as synaptic if within 0.75 μm of the center of FM 4–64 puncta. $GABA_A$R residency time for each cell was given by the mean duration of QD-$GABA_A$R trajectory segments

during which the particle was diffusing within a synapse (defined as above).

To determine AIS and dendrite diameter, confocal images of 25 μm regions (zoom factor 4) were taken. Processes were straightened using the ImageJ Straighten plugin and then thresholded at the same value. To obtain the diameter of the process, the thresholded area was divided by the image length (25 μm).

STATISTICAL ANALYSIS

All experiments were performed on neurons from at least 3 individual preparations. Unless otherwise stated, *p*-values given are from two-tailed Student's *t*-tests (equal variance) and values are given as mean ± s.e.m. Error bars represent s.e.m. For multiple comparisons (i.e., **Figures 5A–C**), One-Way ANOVA followed by Bonferroni correction was used. GABAergic synapse position along axons and $GABA_AR$ diffusion coefficients were not normally-distributed. Differences between conditions in these quantities were tested using the non-parametric Mann-Whitney *U*-test (implemented in *R*).

RESULTS

$GABA_AR$ CLUSTERING AND LATERAL MOBILITY AT THE AXON INITIAL SEGMENT

Subunit composition of $GABA_ARs$ is key to determining their subcellular localization. Previous studies have shown that $GABA_ARs$ containing the α2 subunit are preferentially targeted to the AIS compared to those containing the α1 subunit (Nusser et al., 1996) but whether this is due to subunit-specific differences in receptor diffusion dynamics remains unknown. We investigated the surface clustering and diffusion dynamics of $GABA_ARs$ at the AIS containing either the α1 or α2 subunits using both immunofluorescence and single particle tracking. We surface stained with antibodies to either α1 or α2 subunits and co-stained for ankG to mark the AIS. Both α1 and α2 subunits were found clustered in dendrites as previously described (Nusser et al., 1996; Brünig et al., 2001). $GABA_ARs$ containing the α2 subunit were also routinely found in clusters along axons (**Figure 1A**), while α1 was seen to be more diffuse, but exhibited a clustered distribution in approximately 20% of neurons, (which are likely interneurons,

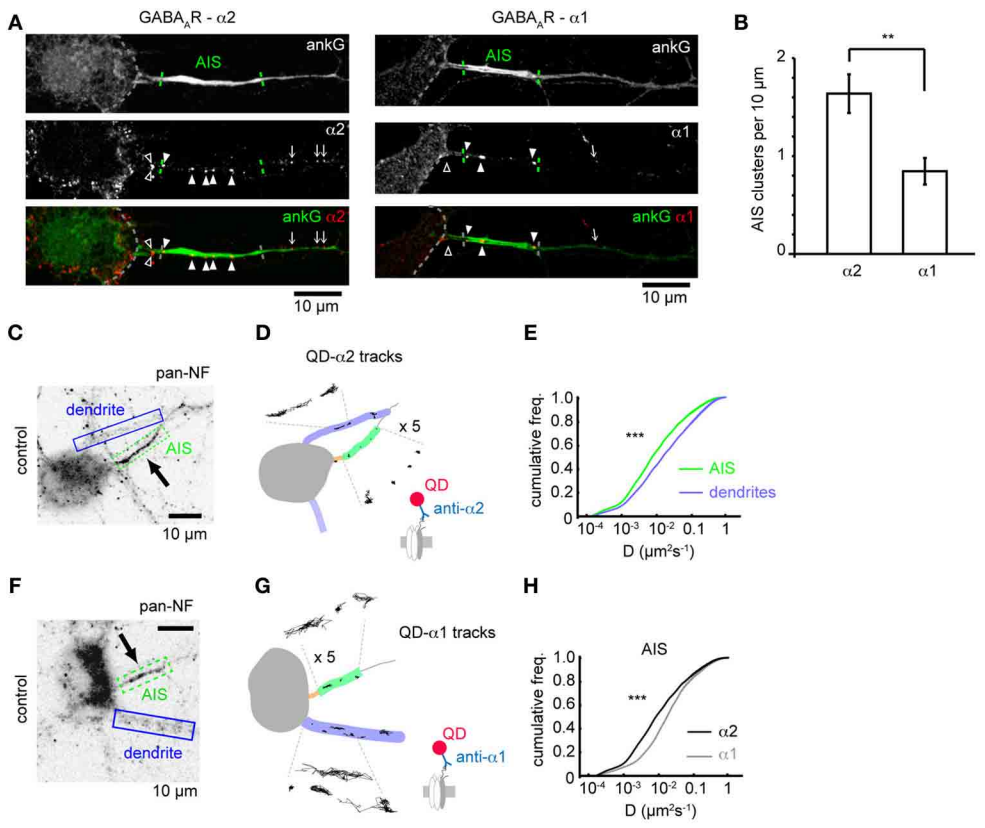

FIGURE 1 | $GABA_AR$ clustering and lateral mobility at the axon initial segment. (A) Example neurons stained for ankG and the $GABA_AR$ α2 (left) and α1 subunits (right). Outline of soma, AIS position and gephyrin cluster positions along the axon are indicated (open arrowhead, cluster before AIS; closed arrowhead, cluster within AIS; arrow, cluster beyond AIS). **(B)** Clusters (normalized per 10μM of AIS length) of the α2 subunit are more numerous in the AIS than the clusters of the α1 subunit, ($p = 0.01$, $n = 5$ experiments). **(C)** Neurofascin (pan-NF) staining delineates the AIS (green). Dendrites (blue) are also identified. **(D)** Subdomains in **(C)** overlaid with QD-α2 tracks (also shown zoomed in × 5 for clarity). **(E)** Cumulative frequency plots of instantaneous α2-$GABA_AR$ diffusion coefficient in AIS (green, $n = 40,681$) and dendrites (blue, $n = 316,774$); 97 cells, 13 experiments, ($p < 2 \times 10^{-16}$). **(F,G)** As in **(C,D)** for QD-α1 labeling. **(H)** α1- and α2-$GABA_AR$ diffusion coefficients in the AIS (gray, α1, $n = 10,999$, 45 cells; black, α2, $n = 40,681$, 97 cells, 13 experiments). α2-$GABA_ARs$ are less mobile in the AIS than α1-$GABA_ARs$ (median D; α2 = 0.008 $\mu m^2 s^{-1}$, α1 = 0.014 $\mu m^2 s^{-1}$, $p < 2 \times 10^{-16}$).

Brünig et al., 2001). In these cells, limited α1 clustering along axons could be seen (**Figure 1A**). In agreement with the literature (Nusser et al., 1996), we found that α2 clusters were far more numerous in the AIS than α1 clusters (α2: 3.6 ± 0.4, α1: 1.6 ± 0.3, $p = 0.002$, **Figure 1B**; quantification was from neurons exhibiting clustered $GABA_AR$ distribution only), confirming that the α2 subunit is enriched at the AIS compared to α1.

We then used single-particle tracking with quantum dots to investigate the lateral mobility of $GABA_ARs$, combined with neurofascin live-labeling (Schafer et al., 2009), to mark the AIS, which reliably labels the AIS as seen by comparison with ankG-GFP expression (**Supplementary Figure 1**). As expected, QD-α2-$GABA_AR$ labeling was seen in the AIS, axon and somatodendritic region (**Figures 1C,D**). Interestingly, α2-$GABA_AR$ lateral mobility was much lower in the AIS than in dendrites (AIS: median $D = 0.008\,\mu m^2 s^{-1}$, dendrites: median $D = 0.016$, $p < 2 \times 10^{-16}$, **Figure 1E**), as has previously been observed for lipid diffusion (Nakada et al., 2003). Recent studies suggest that the diameter of a tubular membrane can affect diffusion measurements (Renner et al., 2011). To assess whether differences in AIS and dendrite diameter could explain the difference in $GABA_AR$ diffusion between these two compartments, we used transfection of membrane GFP (mGFP) and ankG immunostaining to quantify AIS and dendrite diameter (**Supplementary Figure 2**). We found that typical AIS and dendrite diameters were both approximately 1 μm, and were not significantly different ($p = 0.5$), confirming that different tubular diameter could not account for observed differences in AIS and dendritic $GABA_AR$ diffusion.

We also analysed the diffusion dynamics of $GABA_ARs$ containing the α1 subunit (**Figures 1F,G**). Interestingly, while α1-$GABA_ARs$ were less mobile at the AIS than dendrites (AIS: median $D = 0.014\,\mu m^2 s^{-1}$, dendrites: median $D = 0.022\,\mu m^2 s^{-1}$), they were much more mobile than α2-$GABA_ARs$, particularly at the AIS (median D; $\alpha1 = 0.014\,\mu m^2 s^{-1}$, $\alpha2 = 0.008\,\mu m^2 s^{-1}$, $p < 2 \times 10^{-16}$, Mann-Whitney *U*-test, **Figure 1H**), but also in dendrites (median D; $\alpha1 = 0.022\,\mu m^2 s^{-1}$, $\alpha2 = 0.016\,\mu m^2 s^{-1}$). Using the ratio of median *D*-values (dendrite/AIS) as a measure of lateral mobility restriction in the AIS suggests that α2-$GABA_ARs$ are more stable in the AIS membrane than their α1-containing counterparts (median D_{dend}/D_{AIS}: α1 = 1.6, α2 = 2.0), which likely underpins the enriched expression of α2 subunit-containing $GABA_ARs$ observed in this region.

THE AIS SHIFTS DISTALLY ON CHRONIC DEPOLARIZATION, BUT GABAergic SYNAPSE POSITIONS ARE NOT AFFECTED

Recent studies have shown that the AIS can undergo activity-dependent structural plasticity (Kole and Stuart, 2012), and that the AIS can shift distally along the axon in response to chronic depolarization (Grubb and Burrone, 2010). However, whether GABAergic synapses made onto axons (at axo-axonic synapses) also move distally, or adapt to changes in activity, remains unknown. We studied these synapses both under control conditions and after chronic depolarization (15 mM KCl, 48 h, Grubb and Burrone, 2010) by using immunostaining for key GABAergic synapse components (α2, gephyrin, VGAT) and co-staining with ankG (**Figures 2A,A′**). As previously demonstrated (Grubb and Burrone, 2010), chronic depolarization caused a distal shift in AIS start position (control AIS start position: 8.0 ± 0.8 μm from soma, KCl: 12.3 ± 0.9 μm, $p = 0.002$, **Figure 2B**) while AIS length was unaffected ($p > 0.05$, **Figure 2B′**). In contrast to AIS translocation, we found no difference in the number (control: 8.4 ± 0.6; KCl: 8.7 ± 0.6, $p > 0.05$, **Figure 2C**), or position of α2-$GABA_ARs$ clusters along axons between control and KCl-treated neurons (**Figures 2D,E**, $p > 0.05$). In agreement with this, a significant decrease in the ratio of axonal α2-$GABA_AR$ clusters in AIS/before AIS was seen (control: 2.0 ± 0.2; KCl: 1.2 ± 0.1, $p = 0.004$, **Figure 2F**), further suggesting that α2-$GABA_AR$ cluster positions along the axon remain fixed compared to homeostatic AIS repositioning.

Gephyrin, a key scaffold protein of $GABA_ARs$ at synapses, is also clustered at AIS synapses (Panzanelli et al., 2011). Immunostaining for gephyrin and ankG showed that gephyrin formed numerous clusters along the AIS, and also further along axons (**Figure 2G**). As for $GABA_AR$ clusters, we found that the number (**Figure 2H**) and position (**Figure 2I**) of gephyrin scaffolds was unaffected by chronic depolarization, leading to a significant decrease in the ratio of gephyrin clusters in AIS/before AIS (**Figure 2J**). We then used staining for the vesicular GABA transporter VGAT to investigate whether the positioning of presynaptic terminals along the axon was similarly unaffected by chronic depolarization. Similarly, we found no change in the position of GABAergic presynaptic terminals (**Figures 2K–N**). To confirm that clusters of GABAergic synaptic components found along axons represented bona fide GABAergic synapses, we performed co-labeling for $GABA_ARs$ (γ2 subunit) and VGAT (**Supplementary Figure 3**). We found that a high proportion (85%) of $GABA_AR$ clusters along the axon were closely opposed to VGAT clusters, and that this value was similar to that for $GABA_AR$ clusters along dendrites, confirming that GABAergic synapses form along axons. Taken together, these results suggest that the entire GABAergic synapse remains fixed in position during chronic depolarization, and that the tight pre-post coupling of GABAergic synapses (Dobie and Craig, 2011) along the axon is not significantly disrupted during AIS structural plasticity.

CHANGES IN $GABA_AR$ CLUSTER SIZE AND LATERAL MOBILITY AT THE AIS IN RESPONSE TO CHRONIC DEPOLARIZATION

Using confocal microscopy we then examined $GABA_AR$ cluster size in the AIS and dendrites on chronic depolarization. Under control conditions, $GABA_AR$ clusters were larger in the AIS than dendrites (mean size: AIS, 0.145 ± 0.007 μm^2; dendrites, 0.087 ± 0.013 μm^2, $p = 0.0002$, **Figures 3A,A′**). On chronic depolarization, a small but significant decrease in cluster size was seen in the AIS (control: 0.145 ± 0.007, KCl, 0.124 ± 0.004 μm^2, $p = 0.03$, **Figure 3B**). However, dendritic cluster size was slightly but not significantly increased (control, 0.087 ± 0.013, KCl, 0.095 ± 0.006 μm^2, $p = 0.2$, **Figure 3C**). Thus, while the position of pre and postsynaptic elements of the GABAergic synapse are uncoupled from activity-dependent AIS translocation, chronic activity drives an AIS-specific reduction in the postsynaptic size of GABAergic synapses.

To investigate if the alteration in α2-$GABA_AR$ AIS cluster size after chronic activity is due to altered $GABA_AR$ diffusion

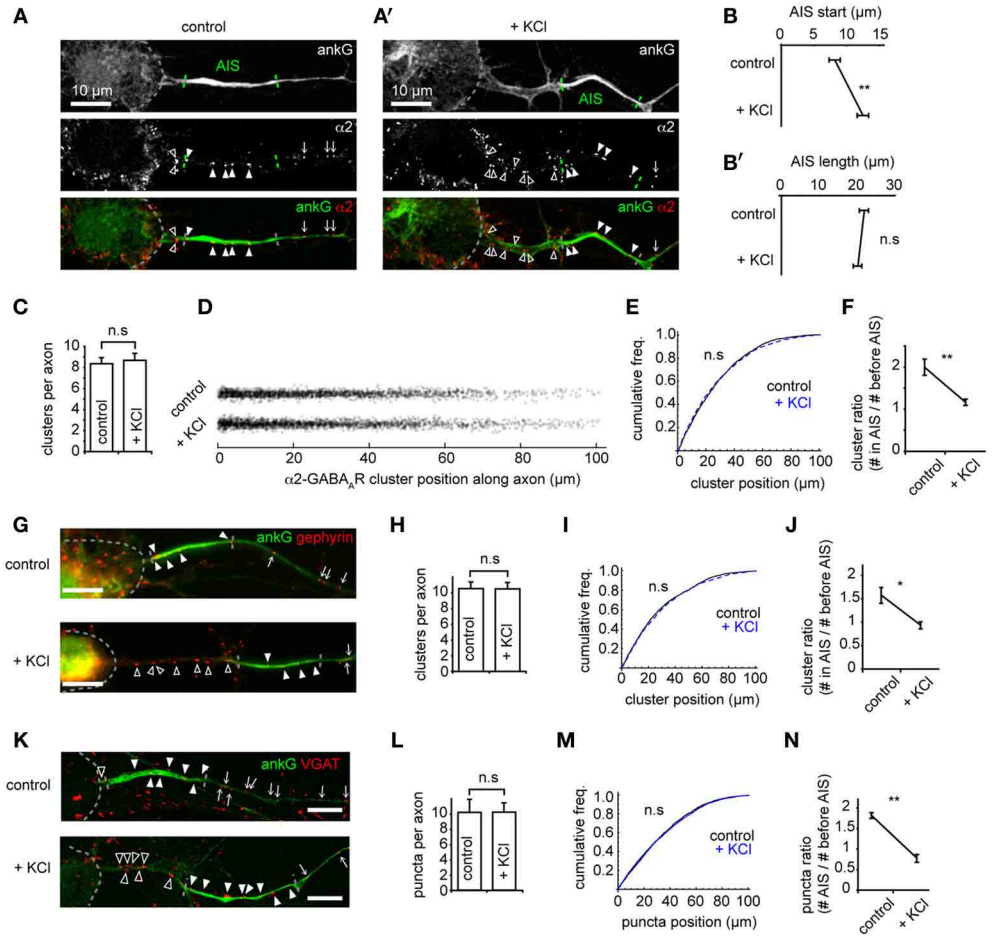

FIGURE 2 | The AIS shifts distally on chronic depolarization, but GABAergic synapse positions are not affected. (A) As **Figure 1A**. Neuron stained for ankG and GABA$_A$R-α2 subunit. Soma, AIS and GABA$_A$R cluster positions along the axon are indicated (open arrowhead: cluster before AIS; closed arrowhead: cluster within AIS; arrow: cluster beyond AIS). AIS endpoints are determined from ankG intensity. **(A′)** as (**A**), for a KCl-treated neuron. **(B)** AIS start position is greater after KCl treatment ($p = 0.007$, $n = 10$ experiments); **(B′)** AIS length is not affected ($p > 0.05$). **(C)** Number of GABA$_A$R clusters per axon is unchanged ($p > 0.05$). **(D)** Positions of all GABA$_A$R clusters from control and KCl pools. **(E)** GABA$_A$R cluster position along axons (control, black, $n = 1944$ clusters; KCl, blue, dashed, $n = 2130$ clusters, $p > 0.05$, Mann-Whitney *U*-test). **(F)** Cluster ratio (in AIS / before AIS) is lower in KCl-treated neurons, $p = 0.004$, $n = 5$ experiments. **(G)** Example neurons stained for ankG and gephyrin (labeled as in **A**). Top: control, bottom: KCl. Scale bar = 10 µm. **(H)** Total number of gephyrin clusters per axon is not significantly different ($p > 0.05$, $n = 5$). **(I)** Cumulative frequency plot of gephyrin cluster position along axons (control, black, $n = 1585$ clusters; KCl, blue, dashed, $n = 1686$ clusters). Distributions not significantly different ($p > 0.05$). **(J)** Gephyrin cluster ratio (in AIS/before AIS) is reduced in KCl-treated neurons, (control: 1.9 ± 0.1; KCl: 0.8 ± 0.1, $p = 0.008$, $n = 5$ preps). Under control conditions, axons contained on average 2.5 ± 0.4 gephyrin clusters before their AIS and 3.8 ± 0.5 clusters within their AIS; in KCl treated neurons, axons contained 3.6 ± 0.6 clusters before their AIS and 3.2 ± 0.5 within their AIS. **(K)** Example neuron stained for ankG and VGAT (labeled as in **A**). Top: control, bottom, KCl. Scale bar = 10 µm. **(L)** Total number of VGAT puncta per axon is not significantly different ($p > 0.05$, $n = 5$). **(M)** Cumulative frequency plot of VGAT puncta position along axon (control, black, $n = 2219$ clusters; KCl, blue, dashed, $n = 1962$ clusters). Distributions not significantly different ($p > 0.05$), suggesting that the tight coupling between pre- and post-inhibitory synapses is not affected by chronic depolarization. **(N)** Cluster ratio (in AIS/before AIS) is lower in KCl treated neurons, (control: 1.9 ± 0.1; KCl: 0.8 ± 0.1, $p = 0.003$, $n = 5$ experiments). Under control conditions, axons contained on average 1.7 ± 0.3 VGAT puncta before their AIS and 3.1 ± 0.5 puncta within their AIS; in KCl treated neurons, axons contained 3.3 ± 0.2 puncta before their AIS and 2.7 ± 0.5 within their AIS.

dynamics in the AIS, we compared GABA$_A$R diffusion dynamics between control and chronically depolarized conditions (**Figures 3D–G**). Chronic depolarization led to a striking increase in GABA$_A$R lateral mobility in the AIS (control-AIS: median $D = 0.008\,\mu m^2 s^{-1}$, KCl-AIS: $0.016\,\mu m^2 s^{-1}$, $p < 2 \times 10^{-16}$, **Figures 3H,K**), and GABA$_A$R diffusion rates also increased in the proximal axon (between soma and AIS start, control-PA: median $D = 0.009\,\mu m^2 s^{-1}$, KCl-PA: $0.015\,\mu m^2 s^{-1}$, $p < 2 \times 10^{-16}$, **Figures 3I,K**). In contrast, GABA$_A$R lateral mobility in dendrites was unaffected (control: median $D = 0.016\,\mu m^2 s^{-1}$, KCl: $0.017\,\mu m^2 s^{-1}$, $p > 0.05$, **Figures 3J,K**). These data suggest that chronic depolarization has a subdomain-specific effect on α2-GABA$_A$R diffusion dynamics. Diffusion dynamics of α1-containing GABA$_A$Rs in the AIS increased only slightly

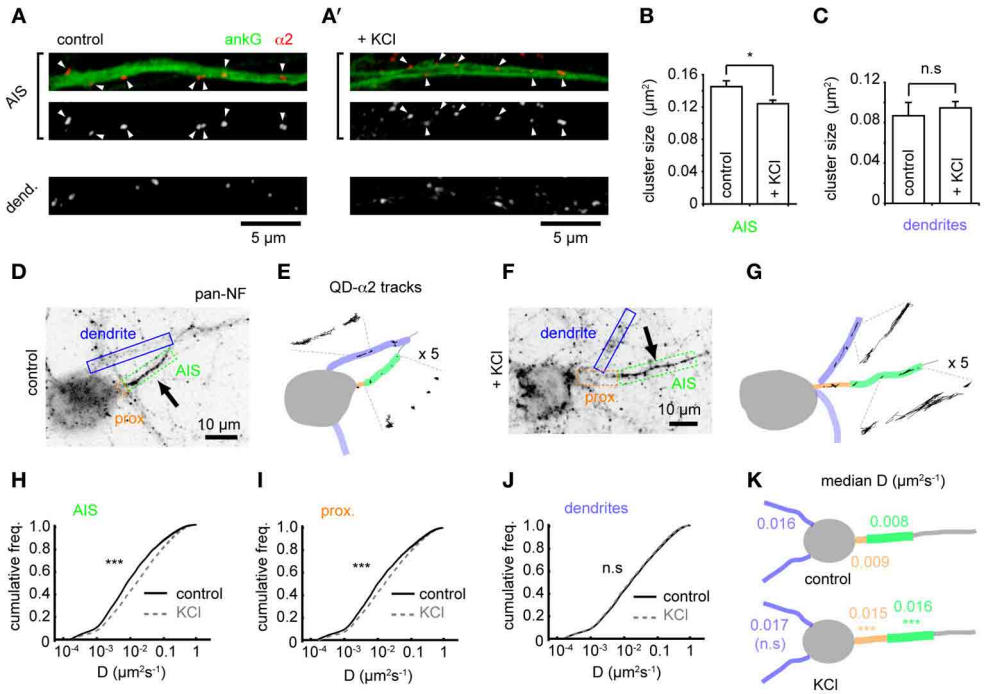

FIGURE 3 | Changes in GABA$_A$R cluster size and lateral mobility at the AIS in response to chronic depolarization. (A) α2 clustering in AIS and dendrites from control neuron. Top panel: AIS, ankG (green), α2 (red). Arrowheads: clusters in AIS. **(A′)** As **(A)**, for KCl-treated neuron. **(B)** α2-GABA$_A$R cluster size in the AIS is decreased by KCl treatment ($p = 0.03$, $n = 5$ experiments). **(C)** α2-GABA$_A$R cluster size in dendrites is unaffected ($p > 0.05$). **(D)** As **Figure 1F**. Neurofascin live labeling delineates the AIS (boxed in green). Proximal axon (orange) and dendrites (blue) can also be identified from background staining. **(E)** Map of subcompartments shown in **(A)**, overlaid with QD- α2 tracks. **(F,G)** As in **(C,D)** for KCl-treated neuron. **(H–J)** Instantaneous α2-GABA$_A$R diffusion coefficient in control (black) and KCl conditions (gray, dashed) in AIS **(H)**, proximal axon **(I)** and dendrites **(J)**. Control, 97 cells; KCl, 111 cells; 10 experiments. In the AIS, median D increased 2-fold, $n_{control} = 40{,}681$, $n_{KCl} = 49{,}235$, $p < 2 \times 10^{-16}$; in the proximal axon, median D increased 1.7-fold, $n_{control} = 11{,}314$, $n_{KCl} = 19{,}979$, $p < 2 \times 10^{-16}$; in dendrites, lateral mobility was unaffected, $n_{control} = 316{,}962$, $n_{KCl} = 463{,}969$, $p > 0.05$. **(K)** Summary of median instantaneous α2-GABA$_A$R diffusion coefficient in control and KCl conditions.

on chronic depolarization, exhibiting a much smaller change than that seen for α2-GABA$_A$Rs in this region (control: median $D = 0.014\,\mu m^2 s^{-1}$, KCl: median $D = 0.017\,\mu m^2 s^{-1}$, $p < 2 \times 10^{-16}$, **Supplementary Figure 4**), and α1-GABA$_A$R lateral mobility was unaffected in dendrites (control: median $D = 0.022\,\mu m^2 s^{-1}$, KCl: median $D = 0.022\,\mu m^2 s^{-1}$, $p > 0.05$). Taken together, these results suggest that the subdomain-specific modulation of GABA$_A$R lateral diffusion in response to chronic depolarization primarily affects α2-containing GABA$_A$Rs.

CHRONIC DEPOLARIZATION AFFECTS SYNAPTIC AND EXTRASYNAPTIC GABA$_A$Rs, AND REDUCES GABA$_A$R RESIDENCY TIME AT AIS SYNAPSES

To investigate whether increased AIS-α2-GABA$_A$R diffusion dynamics altered receptor behavior at synapses, we labeled presynaptic inputs with FM 4–64. Active presynaptic terminals (FM-positive puncta) were routinely found along neurofascin labeled AISs (**Figures 4A,B**). Chronic activity increased α2-GABA$_A$R diffusion both inside and outside synapses made onto the AIS, with similar increases in each domain (median D_{syn} increased 1.8-fold from 0.009 to $0.016\,\mu m^2 s^{-1}$, **Figure 4C**; median D_{ext} increased 1.8-fold from 0.010 to $0.018\,\mu m^2 s^{-1}$, **Figure 4D**, both $p < 2 \times 10^{-16}$). We also analysed the mean time spent by GABA$_A$Rs at synapses. We found that synaptic α2-GABA$_A$R residency time at the AIS was significantly decreased (control: 3.1 ± 0.6 s, KCl: 0.9 ± 0.2 s, $p = 0.001$, **Figure 4E**), suggesting reduced occupancy of synaptic sites, consistent with the decrease in GABA$_A$R cluster size observed above. In contrast, chronic depolarization did not affect GABA$_A$R lateral mobility in dendrites, either at synapses or outside synapses (median D_{syn}, control = $0.013\,\mu m^2 s^{-1}$, KCl = $0.014\,\mu m^2 s^{-1}$; median D_{ext}, control = $0.018\,\mu m^2 s^{-1}$, KCl = $0.018\,\mu m^2 s^{-1}$; both $p > 0.05$, Mann-Whitney U-test, **Figures 4F,G**). Moreover, mean synaptic residency times for GABA$_A$Rs in dendrites were similar between control and KCl conditions (control: 1.6 ± 0.2 s, KCl: 1.5 ± 0.2 s, $p > 0.05$, **Figure 4H**). Taken together, these data further suggest that chronic activity has a region-specific effect on GABA$_A$R diffusion dynamics, with increased diffusion and decreased stability of α2-GABA$_A$Rs at AIS synapses upon chronic depolarization.

DISTAL SHIFT IN AIS POSITION AND INCREASED GABA$_A$R LATERAL MOBILITY DEPEND ON L-TYPE VGCCs

To further understand the mechanisms underlying changes in AIS-GABA$_A$R diffusion dynamics, we investigated the role of L-type voltage-gated Ca^{2+} channels (VGCCs), whose activity was previously reported to drive activity-dependent AIS translocation

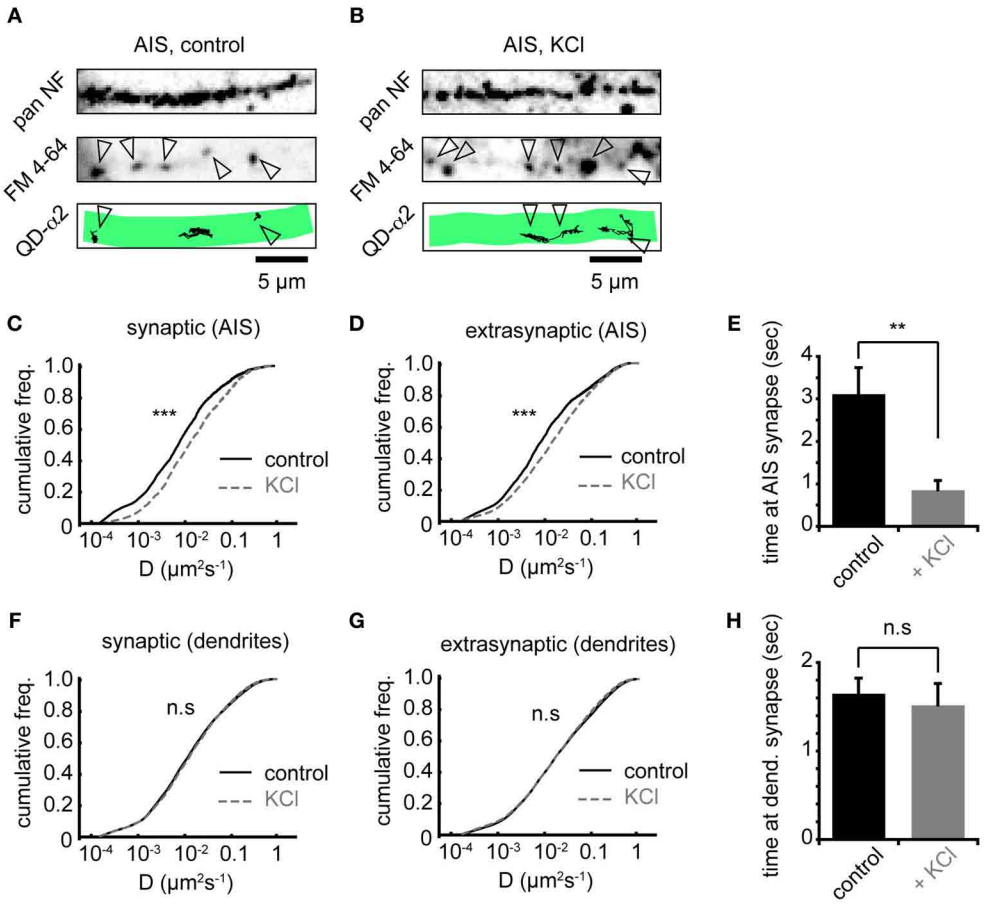

FIGURE 4 | Chronic depolarization affects synaptic and extrasynaptic GABA$_A$Rs, and reduces GABA$_A$R residency time at AIS synapses. **(A)** Control neuron AIS labeled by pan NF (top), FM 4–64 loading (middle, arrowheads = synapses) and with QD-α2 tracks shown (bottom). **(B)** As in **(A)**, but for KCl-treated neuron. **(C)** Chronic depolarization increases GABA$_A$R lateral mobility in the AIS at synapses (1.85-fold increase, $n_{control} = 1922$, $n_{KCl} = 1620$, $p < 2 \times 10^{-16}$). **(D)** GABA$_A$R lateral mobility in the AIS also increases outside synapses (1.89-fold increase, $n_{control} = 4915$, $n_{KCl} = 8077$, $p < 2 \times 10^{-16}$). **(E)** Mean time spent by GABA$_A$Rs at AIS synapses decreases significantly on chronic depolarization (control: $n = 32$ cells; KCl, $n = 26$ cells, $p = 0.001$). **(F,G)** GABA$_A$R lateral mobility in dendrites is unaffected ($p > 0.05$) by chronic depolarization, both in synapses, $n_{control} = 17581$, $n_{KCl} = 13145$ **(F)** and outside synapses, $n_{control} = 75514$, $n_{KCl} = 73135$ **(G)**. **(H)** Mean time spent by GABA$_A$Rs at synapses in dendrites is unaffected by chronic depolarization (control: $n = 32$ cells; KCl, $n = 26$ cells, $p > 0.05$).

(Grubb and Burrone, 2010). We tested whether the L-type calcium channel blocker nifedipine (5 μM) could prevent both the distal AIS shift and the increase in GABA$_A$R lateral mobility at the AIS. Immunostaining for ankG confirmed that blockade of L-type VGCCs could indeed prevent AIS translocation (**Figures 5A–C**). In agreement with the literature, we found that the shift in AIS start position on chronic depolarization was prevented by nifedipine treatment (AIS start position, control: 8.2 ± 1.3 μm; KCl: 14.0 ± 1.2 μm; KCl + nifed: 9.1 ± 0.6 μm, $p < 0.05$ (control vs. KCl), $p < 0.05$ (KCl vs. KCl + nifed), $p > 0.05$ (control vs. KCl + nifed) (**Figure 5B**). Moreover, no change in AIS length was found under either condition (control: 22.8 ± 1.8 μm; KCl: 28.9 ± 2.0 μm; KCl + nifed: 25.8 ± 1.5 μm, $p > 0.05$ for all comparisons, **Figure 5C**). We then analysed α2-GABA$_A$R diffusion dynamics under these conditions (**Figures 5D,D′, E,E′,F,F′**). The robust increase in α2-GABA$_A$R diffusion in the AIS upon chronic depolarization (median D, control: 0.009 μm^{2}s^{-1}; KCl: 0.023 μm^{2}s^{-1}, $p < 2 \times 10^{-16}$, Mann-Whitney U-test) was greatly reduced upon nifedipine treatment (median D, KCl + nif: 0.014 μm^{2}s^{-1}, an 1.64-fold reduction from KCl alone, $p < 2 \times 10^{-16}$, Mann-Whitney U-test, **Figure 5G**). α2-GABA$_A$R lateral mobility in dendrites was similar across control, KCl and KCl + nifedipine conditions (median D control, 0.019; KCl, 0.020; KCl + nif, 0.020 μm^{2}s^{-1}, **Figure 5H**). Thus, Ca^{2+} influx through L-type VGCCs controls both an activity-dependent shift in AIS location and increased AIS-GABA$_A$R lateral mobility.

DISCUSSION

In this study, we have investigated the surface behavior of GABA$_A$Rs at the AIS, both under baseline conditions and in response to changes in neuronal activity that drive AIS structural plasticity (Grubb and Burrone, 2010). We find that surface GABA$_A$Rs are less mobile at the AIS than in dendrites, but that

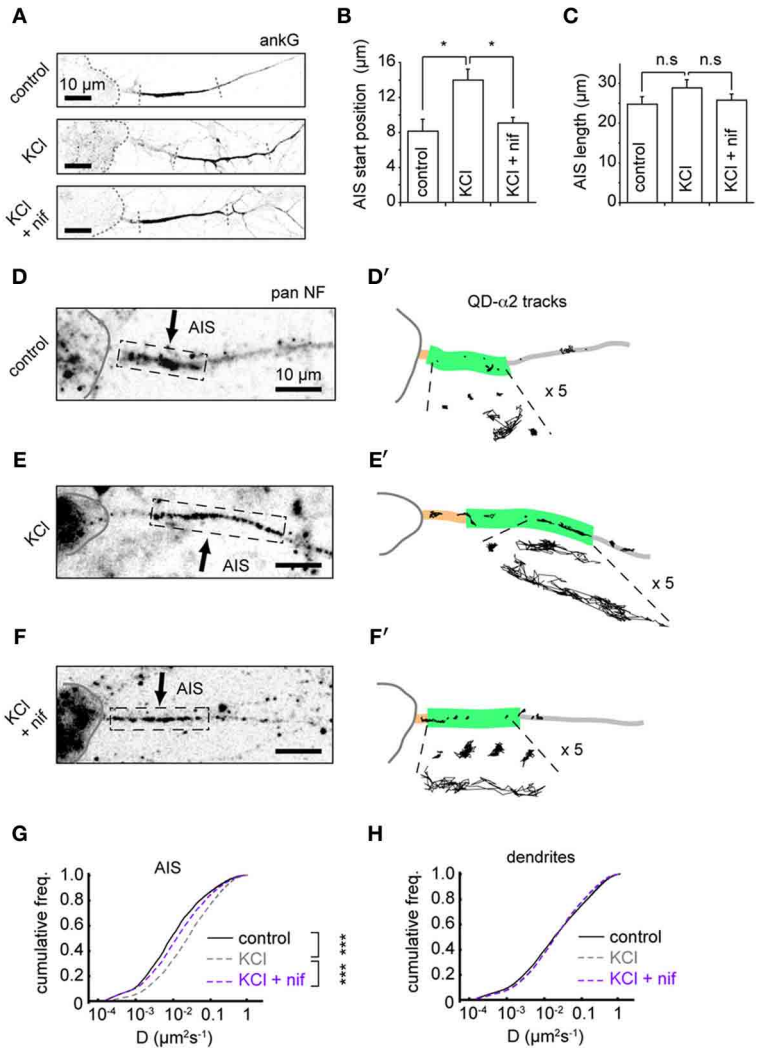

FIGURE 5 | Distal shift in AIS position and increased $GABA_AR$ lateral mobility depend on L-type VGCCs. (A) Example ankG staining from control (top), KCl (middle) and KCl + 5 µM nifedipine conditions (bottom). **(B)** Analysis of AIS start position. One-way ANOVA omnibus test $p = 0.01$. Pairwise test p-values are Bonferroni-corrected. KCl treatment caused a distal shift in AIS start position ($p < 0.05$), which was prevented by addition of nifedipine ($p < 0.05$), $n = 5$ experiments (control = 150 cells, KCl = 132 cells and KCl+nif = 116 cells). **(C)** Analysis of AIS length. Omnibus $p = 0.28$. No change in AIS length was seen in either KCl or KCl + nifedipine ($p > 0.05$ for both comparisons). **(D,D′)** Example control neuron with AIS location given by pan-NF labeling shown with QD-α2 tracks (those in AIS shown with 5× zoom for clarity). **(E,E′)** As above, for KCl treated neuron. **(F,F′)** For KCl + nifedipine condition. **(G)** Instantaneous $GABA_AR$ diffusion coefficient distributions in the AIS for control (black, $n = 9{,}482$, 17 cells), KCl (gray, dashed, $n = 15{,}605$, 22 cells) and KCl + nif (purple, dashed, $n = 6194$, 12 cells). Increase in AIS $GABA_AR$ lateral mobility seen on KCl treatment ($p < 2 \times 10^{-16}$, Mann-Whitney U-test) was reduced in presence of nifedipine, ($p < 2 \times 10^{-16}$, Mann-Whitney U-test). **(H)** As in **(D)**, but for dendrites. Control, $n = 76{,}005$; KCl, $n = 102{,}445$; KCl + nif, $n = 55{,}821$. Dendritic $GABA_AR$ mobilities are similar across conditions.

chronic depolarization drives increased $GABA_AR$ lateral mobility and decreased synaptic residency time at the AIS. Intriguingly, both the distal shift in AIS position and increase in $GABA_AR$ diffusion dynamics at the AIS depend on L-type VGCC activation, suggesting that these activity-dependent responses are linked.

Virtually nothing is known about the behavior of $GABA_ARs$ at the AIS. Indeed, to our knowledge, this is the first study to look at the surface trafficking of $GABA_ARs$ specifically in the AIS. Our investigation into the clustering and lateral mobility of α1- or α2-containing $GABA_ARs$ revealed interesting differences between receptors containing the two subunits. We find that α2-$GABA_ARs$ are more numerous in the axon (as shown previously by immunogold electron microscopy, Nusser et al., 1996), and are also found distributed further down the axon, detectable in clusters 100 µm away from the soma. Moreover, α2-$GABA_ARs$ are less mobile in the surface membrane than α1-containing $GABA_ARs$, especially at the AIS. Differences in the membrane dynamics of receptors containing these two subunits may be due to $GABA_AR$ targeting mechanisms that are subunit-specific.

We also find that α2-$GABA_ARs$ at the AIS and proximal axon are far less mobile than those in dendrites (which are approximately twice as mobile as their AIS-localized counterparts).

Similarly, $GABA_AR$ cluster size in the AIS is almost twice that in dendrites (**Figures 3A–C**), and $GABA_AR$ residency time at synapses in the AIS is longer than for $GABA_ARs$ in dendrites (**Figures 4E,H**). These findings suggest that α2-$GABA_ARs$ are especially stable at AIS synapses. Interestingly, $GABA_ARs$ also exhibit comparably slower surface dynamics at extrasynaptic sites in the AIS in agreement with the notion that properties of the AIS *per se* may play a role in regulating $GABA_AR$ mobilities in this neuronal subcompartment. Slow surface dynamics at the AIS have been previously reported for lipids (Nakada et al., 2003) and NaV channels (Brachet et al., 2010). This could in part be due to the high density of protein scaffolds and membrane proteins at the AIS (Rasband, 2010). However, it is additionally possible that specific protein interactions between the α2 subunit and ankG or another AIS protein (e.g., neurofascin 186, which can stabilize axo-axonic synapses, Kriebel et al., 2011) may also act as diffusion traps to contribute to the increased stability of $GABA_ARs$ at the AIS (i.e., low diffusion rate, high residency time and cluster size). The gephyrin scaffold can interact directly with the α1, α2, and α3 subunits (Tretter et al., 2008; Mukherjee et al., 2011; Tretter et al., 2011) and forms clusters at the AIS (Panzanelli et al., 2011; also herein, **Figure 2G**) suggesting that a complex between $GABA_ARs$, gephyrin and AIS proteins may also exist in this region.

The distal shift undergone by the AIS in response to chronic depolarization (Grubb and Burrone, 2010; also observed herein) is an intriguing cell biological phenomenon, for which a molecular mechanism remains unclear. It was recently identified that an ankyrin-B based scaffold in the distal axon can define the position of the AIS (Galiano et al., 2012), which could be involved in AIS structural plasticity. Whether creation and insertion of new axon from the soma is required is also currently unknown. In contrast to the movement of the AIS, we find that GABAergic synapses distributed along the axon do not undergo a distal shift, as the positioning of pre- and postsynaptic components tested ($GABA_ARs$, gephyrin, VGAT) was found to be unaffected by chronic depolarization. While it is unclear how this may affect the ability of these inputs to regulate the initiation of APs, one possibility is that the resulting increase in the number of synaptic inputs between the soma and the shifted AIS could lead to higher inhibitory shunt acting on conductances reaching the AIS. This would raise the threshold for AP initiation, counterbalancing the chronic activity stimulus and thus acting homeostatically, in concert with the distal shift in AIS position, which causes increased thresholds for action potential initiation (Grubb and Burrone, 2010; O'Leary et al., 2010). Activity-dependent disruption of scaffolding interactions may underlie the observed increase in $GABA_AR$ diffusion and decrease in $GABA_AR$ cluster size at the AIS. Since the activity-dependent increase in $GABA_AR$ mobility at the AIS is also seen extrasynaptically (in gephyrin negative regions) we think it unlikely that alterations in gephyrin-dependent $GABA_AR$ stabilization are the primary driver of the increase in $GABA_AR$ mobility at the AIS upon chronic depolarization. Rather, a distal shift in AIS position but not GABAergic synapses may uncouple $GABA_ARs$ from mechanisms that contribute to their stabilization in the axonal membrane. An intriguing possibility is that the AIS-specific mechanisms that stabilize $GABA_ARs$ in the axon may be weakened in order to allow GABAergic synapses to remain fixed in position and resist the distal shift of the AIS scaffold (including neurofascin 186) in response to chronic depolarization. Increased $GABA_AR$ diffusion dynamics in the AIS and proximal axon could be a necessary consequence of such reduced tethering, to allow the preservation of GABAergic synaptic positions along the axon. Moreover, these putative interactions could be disrupted by activation of L-type VGCCs, since inhibition of L-type VGCCs with nifedipine blocks translocation of the AIS, and also partially prevents an increase in AIS-$GABA_AR$ diffusion on KCl treatment. Previous studies revealed that acute increases in neuronal activity and spiking (e.g., driven by treatment with 4-AP or glutamate) lead to rapid calcium and calcineurin-dependent $GABA_AR$ de-clustering and increased $GABA_AR$ diffusion dynamics in dendrites (Bannai et al., 2009; Muir et al., 2010). In contrast we found that chronic treatment with low levels of KCl (15 mM, 48 h), which was shown to cause only a small 10mV depolarization of the resting membrane potential and a suppression of spontaneous spiking (Grubb and Burrone, 2010) only increased $GABA_AR$ diffusion at the AIS but not in dendrites, suggesting that mild chronic depolarization (with KCl) cannot drive a sufficient rise in dendritic calcium to activate dendritic calcineurin or alter dendritic $GABA_AR$ stability. Interestingly, chronic KCL-dependent AIS repositioning was also recently demonstrated to be calcineurin-dependent suggesting that these conditions may lead to a selective increase in somatic and/or AIS specific calcineurin activity (Evans et al., 2013). This could also account (perhaps in concert with the localization of a specific scaffold such as AnkG to the proximal axon) for a more localized activity-dependent impact on $GABA_AR$ diffusion in the proximal axon/AIS (rather than throughout the entire axon). It will be interesting to determine in the future if the activity-dependent increase in $GABA_AR$ mobility at the AIS is also dependent on changes in $GABA_AR$ phosphorylation state (Muir et al., 2010).

While GABAergic inputs onto the AIS are ideally localized to control action potential initiation (Kole and Stuart, 2012), the nature of these inputs, i.e., whether they are inhibitory or excitatory, is still unresolved. A body of evidence suggests that GABAergic inputs onto the AIS can be depolarizing in the cortex (Szabadics et al., 2006; Khirug et al., 2008; Kole and Stuart, 2012). This is thought to be due to high expression of the $Na^+/K^+/Cl^-$ cotransporter NKCC1 (Khirug et al., 2008) and absence of the K^+/Cl^- cotransporter KCC2 from the AIS (Hedstrom et al., 2008; Báldi et al., 2010), resulting in a high intracellular $[Cl^-]$ and subsequent depolarization on $GABA_AR$ activation. Thus, reduced $GABA_AR$ cluster size and increased $GABA_AR$ diffusion at AIS synapses in response to chronic depolarization could alternatively represent weakening of depolarizing or excitatory GABAergic inputs. In this case, increased $GABA_AR$ diffusion dynamics would provide a mechanism to weaken depolarizing inputs in a homeostatic response to chronic elevation of activity.

We conclude that during activity-dependent AIS translocation, occurring in response to chronic depolarization, the positions of GABAergic synapses along the axon are unaffected. However, the AIS shift is coupled with plasticity of $GABA_AR$ cluster size and diffusion dynamics at this key neuronal subcompartment. This novel form of plasticity could be important for GABAergic control of information processing in the healthy or diseased brain, for

example in epilepsy, where repeated bursts of activity may lead to structural plasticity of the AIS and of axonal $GABA_AR$ diffusion dynamics.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fncel.2014.00151/abstract

Supplementary Figure 1 | (A) Schematic showing live-labeling of AIS via an antibody to neurofascin. We used an antibody to an extracellular epitope on neurofascin (NF), pre-conjugated to alexa dye. **(B)** Overlap of pan-NF live labeling with AIS as marked by ankG-GFP, confirming that this approach can reliably label the AIS. Scale bar = 10 μm.

Supplementary Figure 2 | (A) mGFP-transfected neuron. Left: ankG staining (AIS); right: mGFP expression, with AIS and sample dendrite labeled. **(B)** Zoomed regions of AIS and dendrite shown boxed in **(A)**. **(C)** Process diameter of AIS and dendrites is not significantly different. AIS: 1.0 ± 0.1 μm ($n = 14$), dendrite: 1.1 ± 0.1 μm ($n = 28$), $p = 0.54$).

Supplementary Figure 3 | (A) Axon from neuron stained for ankG (top, cyan), γ2-$GABA_ARs$ (middle, magenta) and VGAT (bottom, yellow), shown merged beneath. Closed arrowheads indicate position of a $GABA_AR$ cluster opposed to a VGAT cluster; open arrowheads indicate position of a $GABA_AR$ cluster not opposed to a VGAT cluster. **(B)** Synaptic $GABA_AR$ cluster fraction in axons and dendrites is not significantly different. Axon: 0.86 ± 0.03 ($n = 30$), dendrite: 0.80 ± 0.01 ($n = 30$), $p = 0.08$.

Supplementary Figure 4 | (A) As **Figure 1C**. Subcompartments delineated from neurofascin staining overlaid with α1-QD trajectories. **(B)** As **(A)**, for KCl-treated neuron. **(C,D)** Instantaneous α1-$GABA_AR$ diffusion coefficient in control (black) and KCl conditions (gray, dashed) in AIS **(C)** and dendrites **(D)**. Control, 45 cells; KCl, 47 cells; 5 experiments. In the AIS, median D increased 1.2-fold, $n_{control} = 10{,}099$, $n_{KCl} = 18{,}933$, $p < 2 \times 10^{-16}$; in dendrites, lateral mobility was unaffected, $n_{control} = 137{,}377$, $n_{KCl} = 175{,}592$, $p > 0.05$.

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

Received: 29 January 2014; paper pending published: 24 February 2014; accepted: 11 May 2014; published online: 10 June 2014.

*Citation: Muir J and Kittler JT (2014) Plasticity of $GABA_A$ receptor diffusion dynamics at the axon initial segment. Front. Cell. Neurosci. **8**:151. doi: 10.3389/fncel.2014.00151*

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

frontiers in
CELLULAR NEUROSCIENCE

MINI REVIEW ARTICLE
published: 06 June 2014
doi: 10.3389/fncel.2014.00157

Stress-induced plasticity of GABAergic inhibition

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GABAergic neurotransmission is highly plastic, undergoing dynamic alterations in response to changes in the environment, such as following both acute and chronic stress. Stress-induced plasticity of GABAergic inhibition is thought to contribute to changes in neuronal excitability associated with stress, which is particularly relevant for stress-related disorders and seizure susceptibility. Here we review the literature demonstrating several mechanisms altering GABAergic inhibition associated with stress, including brain region-specific alterations in $GABA_A$ receptor ($GABA_AR$) subunit expression, changes in chloride homeostasis, and plasticity at GABAergic synapses. Alterations in the expression of specific $GABA_AR$ subunits have been documented in multiple brain regions associated with acute or chronic stress. In addition, recent work demonstrates stress-induced alterations in GABAergic inhibition resulting from plasticity in intracellular chloride levels. Acute and chronic stress-induced dephosphorylation and downregulation of the K^+/Cl^- co-transporter, KCC2, has been implicated in compromising GABAergic control of corticotropin-releasing hormone (CRH) neurons necessary for mounting the physiological response to stress. Acute stress also unmasks the capacity for both long-term potentiation and long-term depression, in distinct temporal windows, at GABAergic synapses on parvocellular neuroendocrine cells (PNCs) in the paraventricular nucleus (PVN) of the hypothalamus. This review highlights the complexity in the plasticity of GABAergic neurotransmission associated with stress and the relationship to neuronal excitability, including alterations in $GABA_AR$ expression, synaptic plasticity at GABAergic synapses, and changes in chloride homeostasis.

Keywords: GABA, neurosteroids, stress, KCC2, GABAA receptors, GABAAR, THDOC

Alterations in neuronal excitability and seizure susceptibility associated with stress have largely been attributed to changes in GABAergic inhibition. Here we review the literature describing stress-induced alterations in neuronal excitability and seizure susceptibility associated with stress and the role of GABAergic neurotransmission. Alterations in the expression of $GABA_ARs$ and the functional consequences on GABAergic inhibition following both acute and chronic stress in multiple brain regions are discussed. In addition, recent evidence pointing to synaptic plasticity at GABAergic synapses and alterations in intracellular chloride levels resulting in compromised GABAergic inhibition following stress is also highlighted.

STRESS-INDUCED CHANGES IN EXCITABILITY

Anecdotally, it is widely accepted that stress is a precipitating factor for seizures (for review see Maguire and Salpekar, 2013). Patients with epilepsy frequently self-report that stress exacerbates their seizures (Neugebauer et al., 1994; Frucht et al., 2000; Haut et al., 2003, 2007; Nakken et al., 2005; Sperling et al., 2008) and increased cortisol levels are positively correlated with seizure frequency in patients with epilepsy (Galimberti et al., 2005) (for review see Lai and Trimble, 1997; Maguire and Salpekar, 2013). However, the relationship between stress and seizure susceptibility is complex. Broadly speaking, acute stress is thought to be anticonvulsant; whereas, chronic stress is thought to increase seizure susceptibility. This section will review the evidence in animal models documenting changes in seizure susceptibility associated with both acute and chronic stress.

ACUTE STRESS

Swim stress (Soubrie et al., 1980; Pericic et al., 2000, 2001; Reddy and Rogawski, 2002), acute cold stress (de Lima and Rae, 1991), and acute restraint stress (de Lima and Rae, 1991) have been shown to increase seizure threshold (for review see Joels, 2009). The anticonvulsant actions of acute stress are thought to be mediated by the production of stress-derived neurosteroids (for review see Rogawski and Reddy, 2004). In response to stress, deoxycorticosterone (DOC) is released which can be metabolized into the neuroactive derivative, allotetrahydrodeoxycorticosterone (THDOC) which has been demonstrated to exert anticonvulsant actions. Further, DOC itself has also been shown to exhibit anticonvulsant properties, a process which requires neurosteroidogenesis (Reddy and Rogawski, 2002) (for review see Rogawski and Reddy, 2004). The anticonvulsant actions of stress-derived neurosteroids are thought to be mediated by their actions on $GABA_ARs$ (for review see Rogawski and Reddy, 2004), which will be discussed more thoroughly in a later section (Stress, seizure susceptibility, and $GABA_ARs$). In addition, recent studies also demonstrate a role for glucocorticoid receptors in the anticonvulsant action of acute stress on seizure susceptibility

(Maggio and Segal, 2012), suggesting the involvement of multiple pathways regulating excitability in response to acute stress.

These data clearly demonstrate the anticonvulsant effects of acute stress, which is contrary to the notion that stress is associated with increased seizure susceptibility and is a trigger for seizures in patients with epilepsy (Minter, 1979) (for review see Maguire and Salpekar, 2013). Although acute stress may have anticonvulsant effects, chronic stress appears to increase seizure susceptibility in animal models, a topic which will be covered in more detail in the following section.

CHRONIC STRESS

Not many controlled studies have been undertaken to elucidate the impact of chronic stress on seizure susceptibility. However, the few studies that have been conducted suggest that chronic social isolation or restraint stress increase susceptibility to seizures induced with bicuculline, picrotoxin, kainic acid, and kindling (Matsumoto et al., 2003; Chadda and Devaud, 2004; Jones et al., 2013). Numerous factors likely mediate the effects of chronic stress on neuronal excitability and seizure susceptibility, including effects on synaptic transmission, adult neurogenesis, and hippocampal remodeling (for review see McEwen, 1999, 2000). The hippocampus is a particularly vulnerable region to the adverse effects of stress, which may mediate the increased neuronal excitability and seizure susceptibility associated with chronic stress (for review see McEwen, 1999, 2000). Furthermore, chronic social isolation stress has been shown to decrease the production of neurosteroids (Serra et al., 2000; Dong et al., 2001), which have anticonvulsant properties (for review see Rogawski and Reddy, 2004), and, thereby, may also influence neuronal excitability. Furthermore, early life stress has also been demonstrated to increase the excitability of principal neurons in the hippocampus and increase seizure susceptibility (for review see Koe et al., 2009), suggesting long-term effects of chronic stress on network excitability.

In addition to stress, there is also a relationship between psychiatric disorders and epilepsy (for review see Jones and O'Brien, 2013). Individuals with psychiatric disorders have a greater risk for developing epilepsy (Forsgren and Nystrom, 1990; Hesdorffer et al., 2000, 2006) and is associated with a poorer outcome (Hitiris et al., 2007; Kanner et al., 2009; Petrovski et al., 2010) (for review see Jones and O'Brien, 2013). These studies suggest that chronic anxiety and other stress-related disorders may increase the susceptibility for seizures. Further, this evidence suggests a proconvulsant role for chronic stress and highlights the complex relationship between stress and seizure susceptibility.

STRESS, SEIZURE SUSCEPTIBILITY, AND $GABA_A$Rs

$GABA_A$Rs are the primary site of neurosteroid action and likely mediate their anticonvulsant effects (for review see Reddy, 2003). THDOC is produced in response to acute stress, increasing to physiologically-relevant levels which can act on $GABA_A$Rs (Purdy et al., 1991; Barbaccia et al., 1996a). Neurosteroids act preferentially at δ-containing $GABA_A$Rs (Wohlfarth et al., 2002), but at higher concentrations can also act on different $GABA_A$R subtypes (Stell et al., 2003; Belelli et al., 2009). In addition to the direct, positive allosteric modulation of $GABA_A$Rs, neurosteroids can also alter the expression of $GABA_A$Rs (for review see Maguire and Mody, 2009; Mody and Maguire, 2011). In fact, alterations in the expression of $GABA_A$Rs associated with both acute and chronic stress may underlie changes in neuronal excitability and seizure susceptibility which will be discussed in more detail below.

STRESS-INDUCED ALTERATIONS IN GABAergic INHIBITION

Many studies examining the impact of stress on GABAergic signaling have relied on the expression of GABA synthesizing enzymes and the binding of GABA ligands. Fewer follow up studies have focused on changes in specific $GABA_A$R subtypes in specific brain regions and the impact on GABAergic inhibition. Many of these studies have focused on stress-induced changes in GABA in the hippocampus in relation to changes in neuronal excitability (for review see Joels, 2009); however, recent interest has also focused on the role of GABA within the stress neurocircuitry (for review see Gunn et al., 2011), particularly the PVN of the hypothalamus.

ACUTE STRESS

In addition to the direct modulatory effects of acute stress-derived neurosteroids on GABAergic inhibition, acute stress has also been proposed to alter GABAergic inhibition via changes in GABA synthesis, release, and the expression of specific $GABA_A$R subunits (for review see Maguire and Mody, 2009; Mody and Maguire, 2011). Changes in GABA synthesis following acute stress have been suggested from alterations in the expression of GABA (Yoneda et al., 1983; Otero Losada, 1988; Acosta et al., 1993) as well as glutamic acid decarboxylase (GAD) (Yoneda et al., 1983; Otero Losada, 1988; Maroulakou and Stylianopoulou, 1991; Acosta et al., 1993; Bowers et al., 1998), the enzyme responsible for the synthesis of GABA (**Table 1**). GABA levels are decreased in the striatum following acute cold stress (Acosta et al., 1993) and decreased in the olfactory bulb following acute immobilization stress (Otero Losada, 1988). In contrast, GABA levels are increased in the striatum and hypothalamus following acute immobilization stress (Yoneda et al., 1983). GAD expression has been shown to be increased in numerous brain regions following acute thermal stress (Maroulakou and Stylianopoulou, 1991) and acute immobilization/restraint stress (Yoneda et al., 1983; Bowers et al., 1998).

There are also changes in the expression of $GABA_A$Rs following acute stress, evident from changes in the binding of radiolabeled GABA ligands. The binding of [3H]GABA is increased in the forebrain following acute swim stress (Skerritt et al., 1981) and in the striatum following acute immobilization stress (Otero Losada, 1988). In contrast, [3H]GABA binding is decreased in the cortex, hypothalamus and olfactory bulb following acute cold stress (Acosta et al., 1993) and decreased in the olfactory bulb following acute immobilization stress (Otero Losada, 1988). [3H]flunitrazepam binding is increased in the cortex following swim stress (Soubrie et al., 1980; Motohashi et al., 1993; Chadda and Devaud, 2004) and t-[^{35}S]butylbicyclophosphorothionate ([^{35}S]TBPS) binding is increased in the cortex following either CO_2 or foot shock stress (Concas et al., 1988; Barbaccia et al., 1996b). The binding of [3H]Ro-15-1788 is decreased in the hypothalamus, cortex, and cerebellum following acute defeat

Table 1 | Acute stress-induced changes related to GABA.

	Stressor	Change, direction	Brain region	Citation
GABA	Cold stress	Decreased	Corpus striatum	Acosta et al., 1993
	Immobilization stress	Increased	Striatum, hypothalamus	Yoneda et al., 1983
		Decreased	Olfactory bulb	Otero Losada, 1988
		No change	Frontal cortex, hippocampus, medio-basal hypothalamus	Otero Losada, 1988; Yoneda et al., 1983
GAD activity	Cold stress	Decreased	Olfactory bulb'	Acosta et al., 1993
	Thermal stress	Increased	Hypothalamus, hippocampus, striatum, cerebral cortex	Maroulakou and Stylianopoulou, 1991
	Immobilization stress	Increased	Striatum, hypothalamus	Yoneda et al., 1983
GAD67	Acute restraint	Increased	Arcuate nucleus, dorsomedial hypothalamic nucleus, medial preoptic area, BnST, hippocampus	Bowers et al., 1998
GAD65	Acute restraint	Increased	BnST, hippocampus	Bowers et al., 1998
[3H]GABA	Swim stress	Increased	Forebrain	Skerritt et al., 1981
		No change	Cerebellum, cortex, temporal cortex, caudate/putamen, lateral septum, hippocampus, amygdala	Skerritt et al., 1981; Skilbeck et al., 2008
	Cold stress	Decreased	Frontal cerebral cortex, hypothalamus, olfactory bulb	Acosta et al., 1993
	Immobilization stress	Increased	Corpus striatum	Otero Losada, 1988
		Decreased	Olfactory bulb	Otero Losada, 1988
		No change	Frontal cortex, hippocampus, medio-basal hypothalamus	Otero Losada, 1988
	Foot shock	Decreased	Frontal cortex, caudate, cerebellum	Biggio et al., 1981
[3H]diazepam	Swim stress	No change	Forebrain, cerebellum	Skerritt et al., 1981
[3H]flunitrazepam	Cold water swim	Increased	Cortex	Soubrie et al., 1980
		No change	Cerebellum	Soubrie et al., 1980
	Swim stress	Increased	Cerebral cortex	Motohashi et al., 1993
		No change	Hippocampus, cerebellum	Motohashi et al., 1993
	Acute restraint	No change	Cortex	Chadda and Devaud, 2004
[3H]muscimol	Swim stress	No change	Cerebral cortex, hippocampus, cerebellum	Motohashi et al., 1993
[35S]TBPS	CO2 stress	Increased	Cortex	Barbaccia et al., 1996a,b
	Foot shock	Increased	Cortex	Concas et al., 1988
[3H]Ro-15-1788	Acute defeat stress	Increased	Hypothalamus, cortex, cerebellum	Miller et al., 1987
		No change	Midbrain, hippocampus	Miller et al., 1987
	Foot shock	No change	Cortex, hippocampus, striatum, cerebellum, hypothalamus	Drugan et al., 1985
α1	Acute restraint	Decreased	Hippocampus, prefrontal cortex	Zheng et al., 2007
		No change	Striatum	Zheng et al., 2007
γ2	CO2 stress	Decreased	Hippocampus	Maguire and Mody, 2007
δ	CO2 stress	Increased	Hippocampus	Maguire and Mody, 2007
KCC2	Acute restraint	Decreased	PVN	Sarkar et al., 2011
KCC2 P-Ser940	Acute restraint	Decreased	PVN	Sarkar et al., 2011

stress (Miller et al., 1987). However, no changes were observed in [3H]diazepam (Skerritt et al., 1981) or [3H]muscimol binding (Motohashi et al., 1993) (**Table 1**). These data are summarized in **Table 1** and demonstrate the complexity in the plasticity of $GABA_A$Rs associated with acute stress (for review see Skilbeck et al., 2010). In addition, changes in specific $GABA_A$R subunits have been described following acute stress.

Table 1 summarizes changes brain region-specific changes in the expression of specific $GABA_A$R subunits associated with several different models of acute stress. Expression of the $GABA_A$R α1 and γ2 subunits are decreased in the hippocampus and prefrontal cortex following acute restraint stress or CO_2 stress (Maguire and Mody, 2007; Zheng et al., 2007). In contrast, the $GABA_A$R δ subunit expression is increased following acute stress in the hippocampus following CO_2 stress (Maguire and Mody, 2007) (**Table 1**). Functional alterations in GABAergic inhibition following acute stress have also been documented in the hippocampus. Following acute restraint stress, there is an increase in the frequency of spontaneous inhibitory post-synaptic currents (sIPSCs) in CA1 pyramidal cells (Hu et al., 2010). In addition, there is an increase in the tonic GABAergic inhibition recorded in dentate gyrus granule cells (DGGCs) following acute CO_2 inhalation stress, consistent with the increased expression of the $GABA_A$R δ subunit (Maguire and Mody, 2007). Interestingly, some of the alterations in $GABA_A$R subunit expression can be mimicked by treatment with THDOC (Maguire and Mody, 2007), suggesting a role for neurosteroids in stress-induced $GABA_A$R plasticity.

Alterations in GABAergic inhibition have also been observed in other brain regions following acute stress. The frequency, but not amplitude, of sIPSCs is increased in pyramidal neurons in the prefrontal cortex following acute stress (inescapable shock). Interestingly, these changes were prevented if the animal was able to exert some control over the stress (escapable shock) (Varela et al., 2012). The frequency of sIPSCs is also increased in the PVN following high frequency stimulation in slices from mice subjected to acute restraint stress (Inoue et al., 2013), which is dependent upon glucocorticoid receptor activation and retrograde opioid signaling (Wamsteeker Cusulin et al., 2013). The increased frequency of sIPSCs in the PVN following acute stress may be due to the increased burst firing of GABAergic interneurons in the peri-PVN area (Shin et al., 2011). These data demonstrate alterations in GABAergic inhibition in several different brain regions following acute stress which may involve both pre- and post-synaptic mechanisms.

CHRONIC STRESS

Alterations in GABAergic inhibition also occur following chronic stress, although these changes appear to be unique from those observed following acute stress. Changes in GABA synthesis following chronic stress have been suggested from alterations in the expression of GABA, GAD65, and GAD67 (**Table 2**). A reduction in the concentration of GABA was observed in the cortex, hypothalamus, and olfactory bulb following chronic cold stress (Acosta et al., 1993). GAD expression is decreased in the striatum and olfactory bulb following chronic cold stress (Acosta et al., 1993); whereas, GAD65 and GAD67 expression are increased in numerous brain regions following chronic intermittent stress (Bowers et al., 1998) and unchanged following repeated swim stress (Montpied et al., 1993). Changes in the expression of $GABA_A$Rs following chronic stress have also been suggested from changes in the binding of radio-labeled GABA ligands. [3H]GABA binding is decreased in the hypothalamus following chronic cold stress (Acosta et al., 1993). The binding of [3H]flunitrazepam is decreased in the frontal cortex following chronic foot shock stress and increased following chronic immobilization stress (Braestrup et al., 1979). No change in [3H]flunitrazepam or [3H]muscimol binding was observed following repeated swim stress (Braestrup et al., 1979; Motohashi et al., 1993). These data are summarized in **Table 2**. Changes in specific $GABA_A$R subunits have also been described following chronic stress.

A decrease in the expression of the $GABA_A$R α1 subunit has been observed in the hippocampus following repeated swim stress with no change observed in α2 or α3 (Montpied et al., 1993). Similarly, a decrease in the expression of the $GABA_A$R β2 subunit was observed in the hippocampus following chronic unpredictable stress (Cullinan and Wolfe, 2000). An upregulation of α5 and β1 subunits have been observed in the PVN following chronic unpredictable stress (Cullinan and Wolfe, 2000; Verkuyl et al., 2004); whereas, a decrease in the expression of the δ subunit was observed in the PVN with no change in α1, α3, α4, γ1, γ2, γ3, or π expression (Montpied et al., 1993; Verkuyl et al., 2004). Chronic social isolation stress results in a decrease in α1 and α2 expression and an increase in α4 and α5 expression in the frontal cortex (Matsumoto et al., 2007). Similarly, an increase in α4 and δ subunit expression has been observed in the hippocampus following chronic social isolation stress (Serra et al., 2006). These changes are summarized in **Table 2**, highlighting the brain region-specific alterations in $GABA_A$Rs following chronic stress. Consistent with changes in $GABA_A$R subunit expression, functional changes in GABAergic inhibition have been observed following chronic stress. An increase in the frequency of sIPSCs has been observed in CA1 pyramidal neurons following chronic restraint stress (Hu et al., 2010), which is mediated by glucocorticoid receptor activation (Hu et al., 2010). In contrast, a decrease in the frequency of sIPSCs has been observed following chronic mild stress in DGGCs (Holm et al., 2011), which is consistent with a decrease in the expression of the α1/α2 and γ2 subunits (Matsumoto et al., 2007). Consistent with an upregulation of the $GABA_A$R α4, α5 and δ subunit (Serra et al., 2006; Matsumoto et al., 2007), an increase in tonic GABAergic inhibition has been measured in DGGCs following chronic stress (Serra et al., 2008; Holm et al., 2011). In the PVN, a decrease in the frequency of sIPSCs has been observed (Verkuyl et al., 2004), which can be mimicked with exogenous corticosterone (Verkuyl et al., 2005) and reversed with adrenalectomy (Verkuyl and Joels, 2003). These data demonstrate the brain region-specific plasticity in GABAergic neurotransmission associated with chronic stress, which may underlie changes in neuronal excitability but also changes in sensitivity to pharmacological compounds.

Impaired GABAergic inhibition following chronic stress may also result from a decrease in neurosteroid synthesis. A decrease in the production of 3α,5α-tetrahydroprogesterone (3α,5α-THP;

Table 2 | Chronic stress-induced changes related to GABA.

Measure	Stressor	Change/ direction	Brain region	Citation
GABA	Cold stress	Decreased	Frontal cerebral cortex, hypothalamus, and olfactory bulbs	Acosta et al., 1993
GAD	Cold stress	Decreased	Corpus striatum, olfactory bulb	Acosta et al., 1993
	Repeated swim stress (7 day)	No change	Hippocampus	Montpied et al., 1993
	Repeated swim stress (14 day)	No change	Hippocampus	Montpied et al., 1993
GAD67	Chronic intermittent stress	Increased	Medial preoptic area, BnST, hippocampus	Bowers et al., 1998
GAD65	Chronic intermittent stress	Increased	Anterior hypothalamic area, dorsomedial nucleus, medial preoptic area, suprachiasmatic nucleus, BnST, perifornical nucleus, periparaventricular nucleus	Bowers et al., 1998
[3H]GABA	Cold stress	Decreased	Hypothalamus	Acosta et al., 1993
[3H]flunitrazepam	Repeated swim stress	No change	Cerebral cortex, hippocampus, cerebellum, striatum, occipital cortex	Motohashi et al., 1993; Braestrup et al., 1979
	Chronic foot shock	Decreased	Frontal cortex	Braestrup et al., 1979
	Chronic foot shock	No change	Striatum, occipital cortex	Braestrup et al., 1979
	Chronic immobilization stress	Increased	Frontal cortex	Braestrup et al., 1979
	Chronic immobilization stress	No change	Striatum	Braestrup et al., 1979
[3H]muscimol	Repeated swim stress	No change	Cerebral cortex, hippocampus, cerebellum	Motohashi et al., 1993
α1	Repeated swim stress (7 day)	No change	Hippocampus	Montpied et al., 1993
	Repeated swim stress (14 day)	Decreased	Hippocampus	Montpied et al., 1993
	Chronic unpredictable stress	No change	PVN	Verkuyl et al., 2004
	Social isolation	Decreased	Frontal cortex	Matsumoto et al., 2007
α2	Repeated swim stress (7 day)	No change	Hippocampus	Montpied et al., 1993
	Repeated swim stress (14 day)	No change	Hippocampus	Montpied et al., 1993
	Social isolation	Decreased	Frontal cortex	Matsumoto et al., 2007
α3	Repeated swim stress (7 day)	No change	Hippocampus	Montpied et al., 1993
	Repeated swim stress (14 day)	No change	Hippocampus	Montpied et al., 1993
	Chronic unpredictable stress	No change	PVN	Verkuyl et al., 2004
α4	Chronic unpredictable stress	No change	PVN	Verkuyl et al., 2004
	Social isolation	Increased	Frontal cortex	Matsumoto et al., 2007
		Increased	Hippocampus	Serra et al., 2006
α5	Chronic unpredictable stress	Increased	PVN	Verkuyl et al., 2004
	Social isolation	Increased	Frontal cortex	Matsumoto et al., 2007
β1	Chronic unpredictable stress	No change	PVN	Verkuyl et al., 2004
		Increased	PVN	Cullinan and Wolfe, 2000
β2	Chronic unpredictable stress	No change	PVN	Verkuyl et al., 2004
		Increased	PVN	Cullinan and Wolfe, 2000
		Decreased	Hippocampus	Cullinan and Wolfe, 2000
β3	Chronic unpredictable stress	No change	PVN	Verkuyl et al., 2004
δ	Chronic unpredictable stress	Decreased	PVN	Verkuyl et al., 2004
		Increased	Hippocampus	Serra et al., 2006
γ1	Chronic unpredictable stress	No change	PVN	Verkuyl et al., 2004
γ2	Chronic unpredictable stress	No change	PVN	Verkuyl et al., 2004
γ3	Chronic unpredictable stress	No change	PVN	Verkuyl et al., 2004
π	Chronic unpredictable stress	No change	PVN	Verkuyl et al., 2004
KCC2	Chronic defeat stress	Decreased	PVN	Miller and Maguire, 2014
KCC2 P-Ser940	Chronic defeat stress	Decreased	PVN	Miller and Maguire, 2014

allopregnanolone) has been observed following chronic stress (Serra et al., 2000; Dong et al., 2001; Pinna et al., 2003; Matsumoto et al., 2007). Decreased levels of neurosteroids may limit the allosteric modulation of $GABA_A$Rs as well as potentially contribute to the observed changes in $GABA_A$R subunit expression (for review see Maguire and Mody, 2009). The decreased production of endogenous positive modulators of $GABA_A$Rs, combined with alterations in $GABA_A$R subunit expression and decreased binding of GABA ligands, indicates altered GABAergic signaling in multiple brain regions following chronic stress.

STRESS-INDUCED ALTERATIONS IN CHLORIDE HOMEOSTASIS

Effective GABAergic inhibition requires the maintenance of the chloride gradient, which is accomplished by the K^+/Cl^- co-transporter, KCC2, in the adult brain (Rivera et al., 1999, 2005; Payne et al., 2003). Recent studies have begun to investigate the impact of changes in chloride homeostasis on GABAergic inhibition under both physiological and pathological conditions, including following acute and chronic stress.

ACUTE STRESS

Our lab recently demonstrated dynamic changes in GABAergic inhibition in CRH neurons in the PVN following acute restraint stress. CRH neurons are at the apex of control of the hypothalamic-pituitary-adrenal (HPA) axis, which mediates the body's physiological response to stress. Following acute restraint stress, KCC2 is dephosphorylated at residue Ser 940 and downregulated in the PVN (Sarkar et al., 2011) (**Table 1**). Although functional deficits in KCC2 transport have not directly been measured following acute stress, shifts in E_{GABA} and compromised GABAergic control of CRH neurons (Hewitt et al., 2009; Sarkar et al., 2011) are thought to result from the dephosphorylation and downregulation of KCC2, leading to excitatory actions of GABA (Sarkar et al., 2011). The changes in KCC2 and GABAergic inhibition following acute stress are unique to these CRH neurons and we believe are part of the signaling cascade required to mount a rapid, all-or-none response to stress.

CHRONIC STRESS

Alterations in KCC2 in the PVN, such as those observed following acute stress, persist following chronic social defeat stress (Miller and Maguire, 2014). The dephosphorylation and downregulation of KCC2 in the PVN is accompanied by stress-induced elevations in corticosterone throughout the chronic social defeat stress paradigm (Miller and Maguire, 2014). These data are consistent with the role of dephosphorylation and downregulation of KCC2 in the PVN in mounting the physiological response to stress. However, deficits in KCC2 transporter function have not been directly measured following chronic stress and future studies are required to determine the significance of these changes on neuronal excitability. Interestingly, the ability of neurosteroids to potentiate GABAergic inhibition and limit the activity of PNCs is reduced following early life stress due to compromised GABAergic inhibition associated with a shift in E_{GABA} in PNCs (Gunn et al., 2013). Thus, it appears that chronic stress impairs the GABAergic control of PNCs, via downregulation of KCC2, which has significant implications for KCC2 as a therapeutic target. Stress-induced changes in chloride plasticity in other brain regions and the impact on neuronal excitability and seizure susceptibility remains to be explored, but are necessary steps given the interest in targeting KCC2 for therapeutics.

PLASTICITY IN THE GABAergic CONTROL OF THE HPA AXIS

In addition to changes in GABAergic neurotransmission resulting from changes in $GABA_A$R subunit expression and/or chloride plasticity, elegant studies have demonstrated stress-induced synaptic plasticity at GABAergic synapses following acute stress. Acute restraint stress has been demonstrated to unmask the capacity for activity-dependent long-term potentiation at GABAergic synapses (LTP_{GABA}) on PNCs in the PVN, a process which involves the activation of β-adrenergic receptors and an upregulation of mGluR1 receptors (Inoue et al., 2013). This potentiation of GABAergic inhibition has been proposed to overwhelm the chloride extrusion mechanisms (Inoue and Bains, 2014), accomplished by KCC2, which accounts for the collapse in the chloride gradient following stress (Hewitt et al., 2009; Sarkar et al., 2011). Interestingly, there is temporal specificity in the plasticity of GABAergic inhibition on PNCs. At 90 min post-restraint stress, GABAergic synapses on PNCs exhibit long-term depression (LTD_{GABA}), mediated by the actions of glucocorticoids (Wamsteeker Cusulin et al., 2013) which is thought to limit the stress response. These data demonstrate the bidirectional plasticity in GABAergic inhibition on PNCs following acute stress and highlight the importance of synaptic plasticity and the dynamic impact on GABAergic neurotransmission in the regulation of the HPA axis.

CONCLUDING REMARKS

Here we review the role of stress in the plasticity of GABAergic inhibition. In addition to changes in $GABA_A$R subunit expression, recent evidence demonstrates a greater complexity in the plasticity of GABAergic neurotransmission associated with stress, involving changes in chloride homeostasis and synaptic plasticity at GABAergic synapses. These data suggest that there may be additional targets, other than $GABA_A$Rs, for managing the impact of stress on GABAergic neurotransmission which has implications for stress-related disorders and seizure susceptibility.

ACKNOWLEDGMENT

Jamie Maguire was supported by NIH Grant R01 NS073574 and a Research Grant from the Epilepsy Foundation.

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

Received: 30 December 2013; paper pending published: 21 January 2014; accepted: 19 May 2014; published online: 06 June 2014.

*Citation: Maguire J (2014) Stress-induced plasticity of GABAergic inhibition. Front. Cell. Neurosci. **8**:157. doi: 10.3389/fncel.2014.00157*

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

frontiers in
CELLULAR NEUROSCIENCE

REVIEW ARTICLE
published: 26 March 2014
doi: 10.3389/fncel.2014.00091

GABAergic synapses: their plasticity and role in sensory cortex

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The mammalian neocortex is composed of a variety of cell types organized in a highly interconnected circuit. GABAergic neurons account for only about 20% of cortical neurons. However, they show widespread connectivity and a high degree of diversity in morphology, location, electrophysiological properties and gene expression. In addition, distinct populations of inhibitory neurons have different sensory response properties, capacities for plasticity and sensitivities to changes in sensory experience. In this review we summarize experimental evidence regarding the properties of GABAergic neurons in primary sensory cortex. We will discuss how distinct GABAergic neurons and different forms of GABAergic inhibitory plasticity may contribute to shaping sensory cortical circuit activity and function.

Keywords: synaptic plasticity, GABA, inhibitory neurons, sensory cortex, inhibition

INTRODUCTION

The many varieties of synaptic plasticity provide a high degree of flexibility to neural circuits, allowing organisms to adapt to changing environments and learn from their experiences. In primary sensory cortex different forms of synaptic plasticity can be induced by manipulations of sensory drive, some of which are limited to specific developmental windows (Hubel and Wiesel, 1970; Carmignoto and Vicini, 1992; Finnerty et al., 1999; Morales et al., 2002; Hensch, 2004; Maffei and Turrigiano, 2008a; Espinosa and Stryker, 2012; Levelt and Hübener, 2012). While most studies of sensory cortical plasticity have focused on the role of glutamatergic synapses, it is now clear that GABAergic inputs and their plasticity play a fundamental role in cortical circuit refinement throughout life.

GABAergic neurons comprise a variety of cell types with distinct physiological characteristics, anatomical locations and capacities for plasticity (Markram et al., 2004; Ascoli et al., 2008; Caspary et al., 2008; Maffei, 2011; Méndez and Bacci, 2011; Rudy et al., 2011; Vitalis and Rossier, 2011; Taniguchi, 2014). This diversity may be one of the most important features of cortical circuits, as it confers a wide range of possibilities for stabilizing, refining the activity of and setting the state of other neurons and neural circuits (Földy et al., 2004; Santhakumar and Soltesz, 2004). Tools to facilitate the direct investigation of these diverse neurons have only recently become available; therefore, our understanding of the role of GABAergic neurons in cortical circuit function is just beginning.

In this review we will discuss recent findings on the role of GABAergic synaptic transmission and plasticity in sensory cortex. We will focus on the role of local inhibitory circuits; however, it should be noted that long range projecting inhibitory neurons have been identified in neocortex (Germroth et al., 1989). These neurons have only recently begun to be functionally characterized (Melzer et al., 2012), suggesting that much work still needs to be done to fully understand the diversity of connectivity and action of GABAergic neurons in the brain. GABAergic interneurons are involved in sharpening sensory responses and regulating circuit excitability, and they are a *sine qua non* of experience-dependent circuit refinement (Ben-Ari et al., 2004; Heimel et al., 2011; Isaacson and Scanziani, 2011; Maffei, 2011; Levelt and Hübener, 2012; Le Magueresse and Monyer, 2013). We will provide evidence for these functions and discuss possible mechanisms involved, as well as common and unique features of GABAergic synaptic plasticity in different sensory cortices. We will conclude with an attempt to reconcile seemingly discrepant experimental results and suggest issues that in our opinion need to be addressed to push this field forward.

GABAergic INHIBITION AND COMPUTATION IN SENSORY CORTEX

The contribution of inhibition to neural network computation goes beyond that of only a regulator of circuit excitability. Inhibitory neurons form highly interconnected networks of electrically and synaptically coupled neurons, and they have a wide range of anatomical and physiological properties ideally suited for driving broad network synchronization (Kawaguchi and Kubota, 1997; Tamás et al., 1998; Galarreta and Hestrin, 1999; Gibson et al., 1999; Amitai, 2001; Galarreta and Hestrin, 2002; Pfeffer et al., 2013; Taniguchi, 2014). Many individual GABAergic neurons connect broadly to local excitatory neurons and specifically

to local inhibitory neurons, allowing them to exert their influence over large portions of neural circuits (Hestrin and Galarreta, 2005; Oswald et al., 2009; Packer and Yuste, 2011; Fino et al., 2013; Pfeffer et al., 2013). GABAergic inhibitory neurons are, therefore, ideally situated to contribute to the generation of activity associated with wakefulness and cognitive processing (Tamás et al., 2000; Whittington et al., 2000; Oswald et al., 2009).

Inhibitory and excitatory inputs interact dynamically to maintain neural networks in a balanced state that favors neural computations (McCormick, 2002; Haider and McCormick, 2009). They are dynamically coordinated and co-activated during both spontaneous and sensory evoked activity both in acute slice preparations (Adesnik and Scanziani, 2010; Graupner and Reyes, 2013) and *in vivo* (Okun and Lampl, 2008; Adesnik and Scanziani, 2010). The coordination of excitatory and inhibitory inputs is believed to underlie dynamic modifications of functional cortical connectivity, and these rapid changes in functional connectivity may be necessary for sculpting sensory responses (Haider et al., 2007; Haider and McCormick, 2009). According to computational models, coordinated and balanced excitation and inhibition can promote decorrelated network activity, which would favor efficient information processing (Renart et al., 2010).

DIVERSITY OF INHIBITORY NEURONS AND THEIR CONNECTIVITY

Computational models and theories of cortical function often treat inhibitory neurons as a single functional class. However, cortical circuits contain groups of GABAergic neurons that can be distinguished according to their functional properties (Markram et al., 2004; Rudy et al., 2011; Taniguchi, 2014). The heterogeneity of GABAergic neuron subtypes has long hindered our understanding of inhibitory circuits and their specific roles in different aspects of circuit function; therefore, principles for identifying and naming the distinct populations of inhibitory neurons across species are being discussed to facilitate comparisons of results obtained from different species and experimental approaches (Ascoli et al., 2008; DeFelipe et al., 2013). One of the most important features of interneurons is their axonal morphology, which can be used to determine the primary subcellular compartment (axons, dendrites, or soma) that they target for inhibition (Somogyi et al., 1998).

In addition, differential gene expression has become a useful tool for identifying populations of interneurons. Nearly all sensory cortical GABAergic neurons are thought to detectably express one, and only one, of three proteins: the calcium binding protein parvalbumin (PV), the peptide hormone somatostatin (SOM), or the ionotropic serotonin receptor 5HT3aR (Kawaguchi and Kubota, 1997; Lee et al., 2010, 2013; Rudy et al., 2011). Expression of the peptide hormone vasoactive intestinal peptide (VIP) delineates a specific subpopulation of 5HT3aR positive interneurons (Lee et al., 2010; Rudy et al., 2011). Because these markers identify distinct populations and account for nearly all GABAergic neurons, they have provided a useful starting point for the functional investigation of groups of inhibitory neurons in sensory cortex (Rudy et al., 2011); however, there is some overlap in the expression of PV and SOM mRNA, and many more markers have been identified both immunohistochemically and through genetic screens that may help identify important functional subclasses (Cauli et al., 1997, 2000; Markram et al., 2004; Freund and Katona, 2007; Lee et al., 2010; DeFelipe et al., 2013; Taniguchi, 2014).

Interneurons have long been classified by their distinct morphological phenotypes and biophysical properties, and these correspond reasonably well with genetic markers. However, the evidence upon which these classifications are drawn relies on observations made across several species, brain regions and ages. These classifications represent the most common features of interneurons marked by these genes that have been observed, but the heterogeneity within these groups has not been systematically studied, especially across development, cortical layers and cortical regions. Most PV expressing neurons show fast spiking (FS) patterns when depolarized and can be classified morphologically as either basket cells or chandelier cells (Kawaguchi and Kubota, 1993; Wang et al., 2002; Markram et al., 2004; Xu and Callaway, 2009; Rudy et al., 2011). Basket cells have axons that synapse predominantly onto the soma and the perisomatic regions of cortical pyramidal neurons, wrapping around the soma like a basket (Kawaguchi and Kubota, 1997; Tamás et al., 2000). Chandelier cells, with axonal boutons resembling candlesticks or "cartridges" (Szentágothai and Arbib, 1974), are a population of interneurons whose axons predominately contact axon initial segments (Somogyi, 1977). Chandelier cells were originally thought to comprise a homogeneous subpopulation of FS PV positive neurons; however, recent evidence that many chandelier cells do not express PV suggests that two types of functionally distinct chandelier cells could exist (Taniguchi et al., 2013; Taniguchi, 2014). While most PV neurons are FS, depending upon the recording and classification method considered, not all PV neurons are FS and not all FS neurons are PV positive (Gray and McCormick, 1996; Cauli et al., 1997; Markram et al., 2004; Ma et al., 2006; Moore and Wehr, 2013; Taniguchi et al., 2013; but see Rudy et al., 2011).

The largest subgroup of SOM expressing neurons is Martinotti cells, which have regular spiking (RS) action potential widths when depolarized, and vertical axons that preferentially target superficial dendritic tufts (Kawaguchi and Kubota, 1997; Thomson et al., 2002; Wang et al., 2004; Ali and Thomson, 2008). However, SOM expressing synapses have also been observed on soma (Gonchar et al., 2002). In addition, a subgroup of SOM positive neurons has spike widths characteristic of FS cells and extensive axonal arborization within layer 4 (Ma et al., 2006; Miyoshi et al., 2007; Xu et al., 2013). The morphology and subcellular targeting of these neurons has not been fully characterized.

VIP is expressed in cells with morphologies consistent with both dendrite and soma targeting interneurons (Lorén et al., 1979; Morrison et al., 1984; Kawaguchi and Kubota, 1996, 1997; Lee et al., 2010; Miyoshi et al., 2010). 5HTR3aR/VIP- neurons comprise the majority of layer 1 neurons and neurogliaform cells (Lee et al., 2010). Neurogliaform cells form gap junction connections with many other classes of cortical interneurons (Simon et al., 2005). They have been proposed to use primarily volume rather than synaptic transmission of GABA (Oláh et al., 2009); however, they have the capacity to regulate specific portions of neural circuits (Chittajallu et al., 2013).

Beyond preference for targeting specific subcellular compartments, patterns of connectivity within and between interneuron subtypes and pyramidal neurons are beginning to emerge. For example, while PV and SOM positive neurons frequently contact pyramidal neurons, VIP positive neurons rarely inhibit pyramidal neurons (Pfeffer et al., 2013). Instead, VIP neurons predominately inhibit SOM neurons (Lee et al., 2013; Pfeffer et al., 2013; Pi et al., 2013), which in turn target VIP and PV neurons (Pfeffer et al., 2013). This connectivity scheme places VIP neurons in an ideal position to mediate disinhibition of excitatory neurons (Lee et al., 2013; Pi et al., 2013). In the primary input layer of sensory cortex, layer 4, basket cells receive direct thalamic input (Beierlein et al., 2003; Cruikshank et al., 2007) and can therefore shape cortical circuit activation through feedforward control over excitatory neuron activity (Gabernet et al., 2005). In addition to inhibiting excitatory neurons, many PV positive neurons target other PV positive neurons (Galarreta and Hestrin, 1999, 2002; Pfeffer et al., 2013).

The ability to perform experiments *ex vivo* in rodent models has allowed for high resolution analysis of biophysical properties and local connectivity of different populations of inhibitory neurons. For example, it was recently shown that even within the PV positive population of inhibitory neurons in primary visual cortex (V1), distinct biophysical properties (Helm et al., 2013) correlate with the level of expression of the SAP97 scaffold protein (Akgul and Wollmuth, 2013). Since inhibitory neuron populations with distinct biophysical properties, molecular markers, birth dates and birth regions have been identified, it has become possible to label specific subpopulations using genetic approaches to facilitate identification and recording from these groups of inhibitory neurons both *ex vivo* and *in vivo* (Taniguchi et al., 2011; Taniguchi, 2014).

While genetic approaches to marking neurons in intact animals are extremely powerful and promising, some of the strategies to genetically label interneuron populations may interfere with GABAergic transmission (Tamamaki et al., 2003; Wang et al., 2009; see discussion of the GAD67-GFP (Δneo) mouse line below) or mislabel neurons, potentially leading to confounds in the interpretation of the data, or producing contrasting findings (Hu et al., 2013). For example, neurons expressing Cre-dependent genes via the somatostatin promoter (Taniguchi et al., 2011) include a population (up to 5–10% of labeled neurons) of FS PV neurons (Hu et al., 2013; Pfeffer et al., 2013), while this degree of overlap has not been observed using immunohistochemical staining (Lee et al., 2010). In addition, the level of expression of neuronal markers used to identify inhibitory neurons can change during development (Wahle, 1993; Felmy and Schneggenburger, 2004) and/or in an activity-dependent fashion (Gomes da Silva et al., 2010; Martin del Campo et al., 2012; Hou and Yu, 2013), possibly affecting the classification of inhibitory neurons. Similarly, the subcellular compartments targeted by inhibitory synapses are under genetic control and can be modified in an activity dependent manner (Bloodgood et al., 2013). Finally, some functionally distinct population of inhibitory neurons may be distinguishable only by graded levels of gene expression, making them difficult to manipulate with genetically encoded tools alone. Comparing the properties, connectivity and function of inhibitory neurons in different circuits, species and developmental stages will be necessary to distinguish general properties of inhibitory neuron function from specific properties that depend on interneuron subtype, species, region or experimental manipulation. However, the heterogeneity within marked populations and potential plasticity of markers and subcellular targets must be taken into account.

MANIPULATING AND MEASURING THE ACTIVITY OF GABAergic NEURONS *IN VIVO*

Several approaches have been employed to study the activity of GABAergic neurons *in vivo* (**Table 1**). The characteristic narrow spike waveform of FS inhibitory neurons allows them to be identified and separated from RS neurons, which are mostly pyramidal cells, in extracellular and juxtacellular recordings. Because identification of waveforms extracellularly is prone to error (Henze et al., 2000; González-Burgos et al., 2005; Gold et al., 2006), channelrhodopsins (ChR) can be expressed in subsets of interneurons and photoactivated to rapidly induce spikes and more precisely identify units recorded extracellularly (Cardin, 2012; Moore and Wehr, 2013). Multielectrode recording arrays are useful for recording extracellularly from several neurons simultaneously, but many are limited in their ability to record from superficial layers. Whole cell recording with *post-hoc* morphological reconstruction allows for more precise laminar identification than is possible with extracellular recordings, and depolarization induced firing patterns and membrane potential fluctuations can be measured. However, due to the size of interneurons relative to pyramidal neurons and their relative scarcity, this technique has low yield. To improve yield and specificity, recordings can be performed in transgenic animals expressing fluorescent markers in populations of GABAergic neurons. Using two-photon microscopy, patch pipettes can be targeted to neurons either expressing or lacking a specific marker (Liu et al., 2009; Gentet et al., 2010). Florescent labeling of GABAergic neurons can also be combined with calcium imaging, which greatly increases the number of neurons that can be simultaneously recorded from at the expense of resolving precise numbers of action potentials (Sohya et al., 2007; Kerlin et al., 2010). Unfortunately, techniques relying on transgenic animals and two photon microscopy are largely limited to studying the superficial layers of mouse cortex at present.

Whole cell recordings can also be used to measure inhibitory drive postsynaptically. Somatic inhibitory and excitatory currents/conductances can be measured to investigate how inhibition contributes to shaping sensory responses (Anderson et al., 2000). The dynamic reversal potential of a sensory response can also be calculated, allowing inferences to be made about the contribution of excitatory and inhibitory receptors to the response (Crochet et al., 2011; Sachidhanandam et al., 2013). These techniques are limited, however, in that they may fail to fully resolve inputs to distal dendrites. Distal inhibitory inputs may effectively shunt excitatory inputs that would have led to regenerative activity (Gidon and Segev, 2012) without themselves being fully resolved. Functional GABA mediated inhibition has been observed that requires regenerative dendritic activity to be measurable (Palmer et al., 2012). Measuring inhibitory

Table 1 | Approaches for recording the activity of inhibitory neurons and inhibitory postsynaptic responses *in vivo*.

Technique	Cell type identification	Acquirable data	Limitations	Selected references
Extracellular recordings	Spike width (FS vs. RS)	Spikes	Prone to misidentification, some arrays limited to deep layers	Simons, 1978; Swadlow and Weyand, 1987; Henze et al., 2000; González-Burgos et al., 2005; Gold et al., 2006
	Optogenetics	Spikes	Some arrays limited to deep layers	Cardin, 2012; Moore and Wehr, 2013
Juxtacellular recordings	Spike width (FS vs. RS)	Spikes	Low yield, cannot identify RS interneurons	Wu et al., 2008; Liu et al., 2009
	Two-photon guided targeting	Spikes	Superficial layers	Liu et al., 2009; Ma et al., 2010; Runyan et al., 2010
	Reconstruction	Spikes	Low yield	Wu et al., 2008; Ma et al., 2010; Runyan et al., 2010
Whole cell patch of interneurons	Reconstruction, depolarization induced firing patterns	Membrane potential, spikes	Very low yield	Azouz et al., 1997; Zhu and Connors, 1999; Hirsch et al., 2003; Monier et al., 2003
	Two-photon guided targeting	Membrane potential, spikes	Superficial layers, low yield	Gentet et al., 2010, 2012
Calcium dye imaging	Genetic labeling	Changes in calcium concentration	Superficial layers, cannot resolve individual spikes	Sohya et al., 2007; Kerlin et al., 2010
Whole cell patch of pyramidal neurons	Reconstruction, depolarization induced firing patterns	Somatic inhibitory/excitatory conductances, response reversal potentials	May not resolve dendritic inhibition	Anderson et al., 2000; Martinez et al., 2002; Crochet et al., 2011

FS, fast spiking width. RS, regular spiking width.

currents/conductances requires minimizing the contribution of voltage gated channels and would obscure their inhibition. Further, the analysis of inhibitory responses with this approach does not allow experimenters to selectively identify the contribution of distinct inhibitory neuron subgroups to inhibitory synaptic potentials (or currents) evoked by sensory stimuli.

Pharmacology, genetics, optogenetics and alterations in sensory experience have all been used to manipulate GABAergic neurotransmission *in vivo* (**Table 2**). Application of the potent $GABA_A$ receptor agonist muscimol has been used to increase inhibition (Reiter and Stryker, 1988); however, the ubiquity of $GABA_A$ receptors makes it difficult to interpret the results of these experiments as being due specifically to increased synaptic and extrasynaptic inhibition versus the secondary effect of dampening all cortical activity. Benzodiazepines allosterically enhance GABAergic neurotransmission at specific $GABA_A$ receptors with benzodiazepine binding sites (Benke and Möhler, 2006; Gielen et al., 2012) and are therefore a more selective tool for probing inhibitory circuit function. Conversely, GABAergic transmission can be disrupted either by administering GABA receptor antagonists or by impairing GABA synthetic pathways. For example, chronic administration of the GAD antagonist 3-mercaptopropionic acid (3-MPA) lowers cortical GABA (Harauzov et al., 2010). Transgenic mice that have altered expression of proteins related to GABAergic neuron function can be used to study the effects of either developmental or induced changes in GABAergic function (Hanover et al., 1999; Huang et al., 1999; Tamamaki et al., 2003; Fagiolini et al., 2004; Wang et al., 2009; Palmer et al., 2012; Nahmani and Turrigiano, 2014). To study the roles of interneuron groups in sensory processes, the light gated ion channel/pumps ChR and halorhodopsin/archaerhodopsin (HaR/ArchR) can be specifically expressed to excite or inhibit a class of neurons upon photoactivation (Cardin, 2012). While results using photoactivation are certainly interesting, it is unclear how artificially evoked (Cardin, 2012), broad patterns of interneuron activation relate to their physiological activation by sensory stimuli. A bold approach to increasing the specificity of photoactivation is to use single cell electroporation to limit expression to a small number of cells (Cottam et al., 2013).

Alterations in sensory experience that induce changes in GABAergic circuitry can be studied *in vivo* to allow inferences to be made about the effects of the circuit change on sensory responses. These inferences are, of course, limited by the plethora of changes that may be induced in cortical and subcortical circuits: excitatory, inhibitory and neuromodulatory. In this review, we will consider changes in sensory experience in three categories. Sensory deprivation will refer to manipulations that lead to an overall reduction of sensory drive (e.g., binaural hearing loss, dark rearing, or trimming all whiskers on a vibrissal pad).

Table 2 | Tools for manipulating inhibition *in vivo*.

Class	Tools	Examples	Advantages	Limitations	Selected references
Pharmacology	$GABA_A$ receptor agonists	Muscimol		Broadly acting, silences neurons	Reiter and Stryker, 1988
	$GABA_A$ receptor allosteric enhancers	Benzodiazepines and zolpidem	Enhances GABAergic transmission directly	Broadly acting	Fagiolini and Hensch, 2000; Hensch and Stryker, 2004
	$GABA_A$ receptor antagonists	Picrotoxin and bicuculline	Blocks GABAergic transmission directly	Broadly acting	Sillito, 1975; Kyriazi et al., 1996
	GABA synthesis inhibitors	3-MPA		Broadly acting	Harauzov et al., 2010
	Growth factors	IGF-1	Probe signaling cascades	Broadly acting, actions beyond inhibition	Maya-Vetencourt et al., 2012
Genetics	Overexpression	BDNF	Ubiquitous or targeted overexpression	Depend on expression system	Hanover et al., 1999; Huang et al., 1999
	Knockout	GAD65	Full, ubiquitous knockout	Present through development	Fagiolini and Hensch, 2000
	Conditional knockout/knockdown		Targeted	Depends on knockout/knockdown system	
Optogenetics	Photoactivation	ChR	Targets genetic cell classes, good temporal response	Non-physiological stimulation parameters, non-physiological neurotransmission	Atallah et al., 2012; Cardin, 2012; Lee et al., 2012; Wilson et al., 2012
	Photo-inhibition	HaR/ArchR	Targets genetic cell classes	Incomplete inhibition, slow kinetics	Atallah et al., 2012; Gentet et al., 2012; Lee et al., 2013
Sensory experience	Sensory deprivation	Dark rearing, hearing loss, whisker removal	Delays circuit development	Non-specific effects	Benevento et al., 1992; Chittajallu and Isaac, 2010
	Environmental enrichment	Exercise, sensory enrichment	Re-opens critical periods	Non-specific effects	Sale et al., 2007
	Sensory alterations	Monocular Lid suture, selective whisker removal, high frequency hearing loss	Critical period plasticity useful for modeling human disease	Induces complex changes at excitatory and inhibitory synapses	Hubel and Wiesel, 1970; Drew and Feldman, 2009; Yang et al., 2011

3-MPA, 3-mercaptopropionic acid. ChR, channelrhodopsin. HaR, halorhodopsin. ArchR, archaerhodopsin.

This type of manipulation is typically initiated shortly after birth. Deprivation of one sensory modality can alter cortical circuit activity and responsiveness to sensory stimuli even in sensory areas that are not directly driven by the sensory organ that has been manipulated (Zheng et al., 2014). Environmental enrichment will refer adding objects to an animal's cage that provide multisensory stimulation (Sale et al., 2009). Sensory alteration will refer to manipulations that reduce, or fundamentally alter, a specific sensory input, usually asymmetrically, without eliminating it entirely (e.g., monocular lid suture, surgical strabismus, selective hearing ablation and selective whisker removal). When these manipulations are induced during sensitive periods in postnatal development, they often lead to diminished sensory function if later reversed (Hubel and Wiesel, 1970; Carmignoto and Vicini, 1992; Finnerty et al., 1999; Morales et al., 2002; Hensch, 2004; Maffei and Turrigiano, 2008a; Espinosa and Stryker, 2012; Levelt and Hübener, 2012).

GABAergic NEURON SENSORY RESPONSE PROPERTIES

The diversity of genetic, morphological and electrophysiological properties, as well as patterns of connectivity of GABAergic neurons, suggests that different cell types may have distinct functional properties. Studies of GABAergic neurons' receptive field properties have confirmed this hypothesis, yet sometimes with contrasting results. This is not too surprising, given that important functional differences exist between cortical layers and neuronal subtypes, and different recording techniques selectively favor certain layers and interneuron subtypes over others (**Table 1**). Additionally, the laminar distribution and cortical organization of neurons with different response properties differs strikingly between species. Cats have ocular dominance and orientation columns, while rodents and rabbits do not (Hubel and Wiesel, 1963; Tiao and Blakemore, 1976; Murphy and Berman, 1979; Grinvald et al., 1986; Métin et al., 1988; Girman et al., 1999; Schuett et al., 2002; Van Hooser et al., 2005). Cats, rats, and

mice all have different laminar distributions of neurons with simple and complex visual receptive fields (Hubel and Wiesel, 1962; Kelly and Van Essen, 1974; Gilbert, 1977; Gilbert and Wiesel, 1979; Lin et al., 1979; Parnavelas et al., 1983; Niell and Stryker, 2008). These interspecies differences in the gross organization of neuronal response properties raise the possibility that some features of inhibitory neuron response properties may also be species specific.

In cat V1 there is evidence in favor of FS inhibitory neurons being both broadly and narrowly tuned to sensory stimuli (Azouz et al., 1997; Hirsch et al., 2003; Cardin et al., 2007; Nowak et al., 2008). Tuning appears to vary by cortical layer, as FS neurons in the primary thalamorecipient layer, layer 4, are either broadly tuned or untuned (Hirsch et al., 2003; Cardin et al., 2007; Nowak et al., 2008). FS neurons outside layer 4 show tuning similar to RS neurons (Cardin et al., 2007), but are more binocularly driven (Aton et al., 2013). This suggests that inhibition may modulate sensory response properties in a lamina-specific fashion. In awake rabbits, putative layer 4 FS neurons have broader orientation, direction and frequency tuning, are more binocular and are more sensitive to contrast than putative RS neurons (Swadlow and Weyand, 1987; Swadlow, 1988; Zhuang et al., 2013).

In the anesthetized mouse, populations of interneurons identified as either PV positive or FS are more broadly tuned for orientation than both excitatory neurons (Niell and Stryker, 2008; Kuhlman et al., 2011; Zariwala et al., 2011; Atallah et al., 2012; Li et al., 2012b; Cottam et al., 2013; Runyan and Sur, 2013; but see Runyan et al., 2010) and SOM interneurons (Ma et al., 2010; Cottam et al., 2013). FS neurons are also more binocularly responsive than RS neurons (Yazaki-Sugiyama et al., 2009; Kameyama et al., 2010). Within the PV population, however, there is great tuning diversity, with some PV neurons being well tuned (Runyan et al., 2010; Zariwala et al., 2011; Runyan and Sur, 2013). Interestingly, PV neurons with longer dendrites have broader orientation selectivity than those with shorter dendrites (Runyan and Sur, 2013). Some of the heterogeneous aspects of PV neuron tuning may be due to the diversity of morphological groups of neurons expressing PV: basket cells (small and large) and a subgroup of chandelier cells. While both basket and chandelier cells exert powerful control over pyramidal neuron excitability and firing, they receive distinct sets of inputs, at least in somatosensory cortex (Xu and Callaway, 2009). SOM neurons exhibit weaker responses to visual stimuli than either excitatory or PV neurons (Ma et al., 2010) and exhibit sharper population orientation tuning than PV neurons (Ma et al., 2010; Cottam et al., 2013). Using optogenetic photoactivation of a small number of SOM neurons, it was shown that during visual stimulation, SOM neurons are able to more strongly inhibit responses of PV neurons than excitatory neurons. Interestingly, SOM neuron photoactivation sharpened PV neuron tuning (Cottam et al., 2013).

In layer 2/3 of GAD67-GFP (Δneo) mice, GABAergic neurons are only weakly orientation selective (Sohya et al., 2007; Bonin et al., 2011), and have similar binocularity to glutamatergic neurons (Gandhi et al., 2008). In these mice no difference in selectivity exists between FS and RS GABAergic neurons (Liu et al., 2009) or between SOM, PV, or VIP positive neurons (Kerlin et al., 2010). However, GAD67-GFP (Δneo) mice have reduced orientation selectivity of both GABAergic (Runyan et al., 2010) and glutamatergic neurons compared with control strains (Hagihara and Ohki, 2013; but see Liu et al., 2009). GAD67-GFP (Δneo) mice have decreased expression of GAD67 (Wang et al., 2009). Furthermore, their levels of GABA are reduced neonatally and in the adult (Tamamaki et al., 2003). These caveats should be carefully considered when interpreting data obtained using this strain.

In primary auditory cortex (A1) of anesthetized cats, putative FS inhibitory neurons have short response latencies and broad spectral tuning (Atencio and Schreiner, 2008). Similarly, rat layer 4 FS neurons have faster response latencies and broader tuning than layer 4 pyramidal neurons (Wu et al., 2008). In mice, units identified extracellularly as FS have faster response latencies and broader tuning than RS units (Moore and Wehr, 2013). Once subdivided into PV positive and PV negative units, however, PV positive units did not differ in frequency tuning or response latency from PV negative units, but did have higher spontaneous and evoked firing rates. They also had shallower response gain and were less well tuned for stimulus intensity than PV negative units. Very narrowly tuned PV positive neurons were found, but very rarely. These findings were interpreted as evidence for a role of PV neurons in modulating gain and intensity tuning (Moore and Wehr, 2013). Differently, in superficial layers PV neurons were found to be more broadly tuned than either excitatory or SOM positive neurons, while SOM neurons showed delayed responses compared to PV and excitatory neurons (Li et al., 2014b). VIP neurons are also tone responsive; however, their tuning properties have not been characterized (Pi et al., 2013).

In the barrel portion of primary somatosensory cortex (S1) in rats (paralyzed, anesthetized or sedated) and awake rabbits, putative FS inhibitory neurons show broader sensitivity to the direction of whisker deflection and/or to the number of whiskers stimulated than RS units (Simons, 1978; Simons and Carvell, 1989; Swadlow, 1989; Bruno and Simons, 2002; Gabernet et al., 2005; but see Zhu and Connors, 1999). In addition, they show faster and more reliable responses to stimulation than putative excitatory neurons, especially in layer 4 (Simons, 1978; Swadlow, 1989; Armstrong-James et al., 1993; Bruno and Simons, 2002; Swadlow and Gusev, 2002), where both excitatory and inhibitory neurons are driven monosynaptically from the thalamus (Beierlein et al., 2003; Cruikshank et al., 2007). Within rat layer 2/3, chandelier cells have broader receptive fields than other FS interneurons (Zhu et al., 2004). Within layer 1, deep-projecting GABAergic neurons are well tuned and respond to stimuli rapidly, whereas local interneurons respond slowly and are poorly tuned (Zhu and Zhu, 2004). In the hindpaw region of S1, a sizable portion of inhibitory neurons respond to ipsilateral stimulation (Palmer et al., 2012). Differently from rats and rabbits, putative FS inhibitory neurons in layer 4 of mouse barrel cortex show similar angular tuning to RS units (Kwegyir-Afful et al., 2013). In contrast to other cell types in mouse layer 2/3, SOM neurons are active during quite wakefulness and inhibited during sensation (Gentet et al., 2012). The inhibition of SOM neurons in S1 during whisking is dependent upon VIP neurons, which

are driven to increase their activity during whisking by primary motor cortex (Lee et al., 2013).

Few studies have examined the tuning of GABAergic inhibitory interneurons in primary chemosensory cortices. In the primary gustatory cortex (GC) of anesthetized rats, putative FS inhibitory neurons are primarily tuned to one or two (of four) tastants, almost always including NaCl; while RS units are more broadly tuned and may respond several tastants (Yokota et al., 2011). In the primary olfactory cortex (Pir) of anesthetized rats, layer 1 GABAergic neurons are broadly tuned to odors compared to layer 2/3 pyramidal neurons (Poo and Isaacson, 2009).

Finally, recent studies have identified putative inhibitory interneuron involvement in multisensory integration and learning. Inhibitory neurons in layer 2/3 of a mouse visuotactile area between visual and somatosensory cortices exhibit less multisensory enhancement of evoked responses than pyramidal neurons, and their activation suppresses multisensory enhancement of activity in pyramidal neurons (Olcese et al., 2013). In mouse V1, a local inhibitory circuit can be driven by an auditory stimulus (Iurilli et al., 2012), and in GC of awake rats, a few putative FS inhibitory neurons were found that respond to auditory cues that triggered expectation of taste delivery (Samuelsen et al., 2012). Mouse A1 layer 2/3 VIP neurons and layer 1 interneurons inhibit other GABAergic neurons during auditory fear conditioning (Letzkus et al., 2011) and discrimination tasks (Pi et al., 2013), respectively.

While it is clear that there are lamina and species specific interneuron response properties, some common features exist within cell types (**Table 3**). FS and PV neurons, which predominantly provide somatic, perisomatic and axonal inhibition, tend to be broadly and bilaterally tuned, especially within layer 4. Differently, SOM neurons, which predominantly provide dendritic inhibition, tend to be less strongly responsive to, or inhibited by, stimuli, and those that are responsive to stimuli are tuned intermediately compared to PV and pyramidal neurons. It is unclear how FS SOM neurons and RS PV neurons fit into this scheme. Little is known about the tuning properties of 5HT3aR neurons, whether VIP positive or negative; however, the VIP positive subgroup has been implicated in mediating disinhibition during associative learning and active sensation (Lee et al., 2013; Pi et al., 2013). When comparisons have been made between subgroups of these populations, differences in response properties tied to morphology have been observed. Full understanding of the roles of GABAergic neurons, therefore, will require more detailed investigations of these subclasses, as well as further investigation into the function of genetically identified interneuron classes in subgranular layers.

GABAergic INHIBITION AND SENSORY RESPONSES IN EXCITATORY CELLS

The varied response properties of GABAergic neurons make them ideal candidates to modulate the activity of excitatory neurons during sensory processing. *In vivo* pharmacological manipulation of GABAergic transmission alters receptive fields properties in a number of different species and cortical regions, suggesting a role for cortical inhibition in controlling the stimulus preference and receptive field structure of excitatory neurons (Sillito, 1975, 1979; Sillito et al., 1980; Kyriazi et al., 1996; Tremere et al., 2001; Li et al., 2002; Foeller et al., 2005; Jeong et al., 2009; Razak and Fuzessery, 2009; Isaacson and Scanziani, 2011; Katzner et al., 2011). However, the results of grossly disrupting or augmenting inhibition in cortex do not provide evidence that GABAergic neurons dynamically shape receptive fields, and their contribution to shaping the receptive fields of glutamatergic neurons is hotly debated (Isaacson and Scanziani, 2011; Liu et al., 2011; Wu et al., 2011; Priebe and Ferster, 2012). Depending on the location and specificity of inhibitory inputs onto a pyramidal cell, GABAergic neurons with a range of tuning properties could contribute inhibition tuned similar to, more tightly than or more broadly than excitatory inputs to that neuron, or tonic, stimulus invariant inhibition.

There is general agreement that an inhibitory mechanism contributes to pyramidal neurons having narrower spiking receptive fields than subthreshold receptive fields (Priebe and Ferster, 2008; Isaacson and Scanziani, 2011; Tan et al., 2011; Wu et al., 2011). This sharpening effect of inhibition occurs, at least in part, through an "iceberg" effect, where inhibition contributes to the spike threshold, below which changes in membrane potential do not alter firing. This effect can occur independently of the relative tuning widths of inhibitory and excitatory inputs (see Isaacson and Scanziani, 2011). Static changes in the level of inhibition, independent of tuning, could alter the sharpness of the membrane potential dynamics in response to spiking input/output transformation, effecting a change in gain. Inhibitory inputs with different tuning than excitatory inputs, if they exist, would provide additional sharpening (Wu et al., 2008; Liu et al., 2011).

In V1, lateral inhibition could account for many receptive field properties, including orientation tuning, spatial segregation of light and dark receptive subfields, direction selectivity, contrast invariance and suppression. However, it has also been proposed that most of these response properties can be accounted for by a model based on the properties of feedforward, excitatory thalamic inputs that have been observed *in vivo,* coupled with a spike threshold determined in part by inhibitory inputs (Priebe and Ferster, 2012). However, the ability to account for most receptive field properties without lateral inhibition does not rule out a role for lateral inhibition. We will focus our discussion on the evidence for and against lateral inhibition being observed and playing a role in shaping receptive fields.

Inhibitory and excitatory light and dark receptive subfields are largely spatially segregated in the visual cortex of cats (Ferster, 1988; Hirsch et al., 1998; Priebe and Ferster, 2005). The spatial opposition of inhibition and excitation was proposed to allow for linear summation of inputs: at a given location within a receptive field, dark and light stimuli evoke opposite responses (Priebe and Ferster, 2005). Conversely, voltage clamp recordings from simple cells in mice have revealed spatial overlapping of receptive subfields, with inhibitory light and dark subfields similarly centered and lying between slightly segregated excitatory light and dark subfields. This suggests a role for inhibition in the segregation of light and dark receptive fields in mice (Liu et al., 2010).

Orientation tuning of excitatory and inhibitory inputs to pyramidal neurons has been observed to be either similarly tuned (Anderson et al., 2000; Tan et al., 2011) or to have a variety

Table 3 | Tuning width of inhibitory neurons by cell type.

Interneuron class	Sensory cortex	Layers	Tuning	Species	References
FS	V1	4	Broadly tuned	Cat, Rabbit, Mouse	Swadlow and Weyand, 1987; Swadlow, 1988; Cardin et al., 2007; Li et al., 2012b; Zhuang et al., 2013
		4	Mixed	Cat	Hirsch et al., 2003
		2/3, 5/6	Well-tuned	Cat	Cardin et al., 2007
		Mixed	Broadly tuned	Cat, Mouse	Niell and Stryker, 2008; Nowak et al., 2008
		Mixed	Mixed	Cat	Azouz et al., 1997
	A1	4, Mixed	Broadly tuned	Cat, Rat, Mouse	Atencio and Schreiner, 2008; Wu et al., 2008; Moore and Wehr, 2013
	S1	4, Mixed	Broadly tuned	Rat, Rabbit	Simons, 1978; Simons and Carvell, 1989; Swadlow, 1989; Bruno and Simons, 2002; Gabernet et al., 2005
		4, Mixed	Well-tuned	Rat, Mouse	Zhu and Connors, 1999; Kwegyir-Afful et al., 2013
	GC	Mixed	Well-tuned	Rat	Yokota et al., 2011
PV	V1	2/3, 4	Broadly tuned	Mouse	Ma et al., 2010; Kuhlman et al., 2011; Zariwala et al., 2011; Atallah et al., 2012; Cottam et al., 2013; Runyan and Sur, 2013
		2/3	Mixed	Mouse	Runyan et al., 2010; Zariwala et al., 2011; Runyan and Sur, 2013
	A1	2/3	Broadly tuned	Mouse	Li et al., 2014b
		Mixed	Well-tuned	Mouse	Moore and Wehr, 2013
Chandelier	S1	2/3	Broadly tuned	Rat	Zhu et al., 2004
SOM	V1	2/3, 4	Well-tuned	Mouse	Ma et al., 2010; Cottam et al., 2013
	A1	2/3	Well-tuned	Mouse	Li et al., 2014b
VIP	A1		Tone responsive, tuning unknown	Mouse	Pi et al., 2013
Layer 1	S1	1: deep projecting	Well-tuned	Rat	Zhu and Zhu, 2004
	S1	1: local	Broadly tuned	Rat	Zhu and Zhu, 2004
	Pir	1	Broadly tuned	Rat	Poo and Isaacson, 2009

FS, fast spiking width; V1, primary visual cortex; A1, primary auditory cortex; S1, primary somatosensory cortex; GC, primary gustatory cortex; Pir, primary olfactory cortex; PV, parvalbumin positive neurons; SOM, somatostatin positive neurons; VIP, vasoactive intestinal peptide positive neurons.

of tuning schemes (Martinez et al., 2002; Monier et al., 2003) in cat V1. Orientation tunings of inhibition and excitation were found to be similar in layers 2–4 but different in layer 5 (Martinez et al., 2002). In mice, similar tuning (Tan et al., 2011) and tuning of inhibition just broader than excitation (Liu et al., 2011; Li et al., 2012b) have both been reported. To address whether specific subclasses of inhibitory neurons have the capacity to alter orientation tuning, optogenetic stimulation has been used to control the activation of GABAergic neurons during visual stimulation while recording responses in superficial excitatory neurons. Photoactivation of PV neurons preferentially inhibited responses to optimal orientation without changing orientation selectivity (Atallah et al., 2012; Wilson et al., 2012), while photoactivation of SOM neurons inhibited responses at all orientations equally, sharpening orientation selectivity (Wilson et al., 2012). However, in a contrasting study PV neuron photoactivation sharpened orientation selectivity with minimal effects at optimal orientations, while SOM and VIP positive neurons' photoactivation suppressed firing but did not alter orientation tuning (Lee et al., 2012). Further, performance in an orientation discrimination task was improved by selective photoactivation of PV positive neurons (Lee et al., 2012). The differences in results may be due to the stimulation parameters used (Lee et al., 2012). As discussed above, it is unclear how these results relate to physiological roles of inhibitory neurons.

Experiments examining the role of inhibition in sharpening tuning width in sensory areas other than V1 have yielded similarly contrasting results. In rodent A1, frequency tuning of inhibitory inputs has been found to be approximately similar to (Wehr and Zador, 2003; Zhang et al., 2003; Tan et al., 2004; Froemke et al., 2007; Tan and Wehr, 2009; Dorrn et al., 2010; Li et al., 2014a) or slightly broader than (Wu et al., 2008; Sun et al., 2010) excitatory inputs. In addition, intensity tuning of excitatory and inhibitory inputs to non-monotonically tuned

neurons has been observed to be similarly (Wehr and Zador, 2003) and differentially tuned (Wu et al., 2006; Tan et al., 2007). In rat Pir, inhibitory inputs to layer 2/3 pyramidal neurons are broadly tuned to odors (Poo and Isaacson, 2009).

Experimental evidence in favor of a role for lateral inhibition in sharpening the selectivity of tuning curves has been found, but it has not been observed consistently either within species or cortical regions (**Table 4**; Priebe and Ferster, 2012). Possible reasons for these inconsistencies include anesthetic choices, stimulus parameters, age of animals, recording layers and experimental approaches. Recent data from layer 2/3 of mouse V1 demonstrated that in awake mice the ratio of inhibition to excitation observed in response to visual stimuli is much greater than under anesthesia (Haider et al., 2013). Further, strong inhibition could be evoked by stimulation in the receptive field surround only during wakefulness (Haider et al., 2013). Thus, the state of a neuron or network can profoundly affect receptive field properties and alter the function of inhibition within a circuit. In layer 4 of mouse V1, increasing the contrast of a grating stimulus broadens inhibitory tuning, leading to contrast-dependent sharpening of orientation selectivity (Li et al., 2012a). Age-dependent sharpening (Dorrn et al., 2010) and widening (Sun et al., 2010) of inhibitory tunings have been reported in rat A1. Developmental broadening of inhibition, both in the tuning of PV neurons and in inhibitory inputs to pyramidal cells has been observed in mouse V1 (Kuhlman et al., 2011; Li et al., 2012b). Therefore, there may be specific conditions and developmental windows in which lateral inhibition contributes substantially to sharpening neuronal receptive fields. A neural network computational model has been developed that can operate in both co-tuned and lateral inhibition modes, depending upon the input received (Levy and Reyes, 2011), and another set of theoretical models suggests that the diversity of receptive fields seen *in vivo* may be accounted for by the existence of multiple network architectures existing simultaneously (de la Rocha et al., 2008; Piëch et al., 2013). These results, coupled with the limitations of recording inhibition somatically, make definitive conclusions against a role for lateral inhibition in sharpening response selectivity tenuous.

The relative timing of excitatory and inhibitory inputs to a neuron can influence its ability to integrate inputs. Inhibition that follows excitation with a brief delay can create a narrow temporal window within which spiking can occur (Wehr and Zador, 2003; Wilent and Contreras, 2004; Higley and Contreras, 2006). If the delay before the onset of inhibition varies with the stimulus, this delay could participate in coding. In rat S1, excitatory and inhibitory inputs to layer 3 and 4 neurons are poorly tuned

Table 4 | Relative tuning of excitatory and inhibitory somatic conductances/currents in excitatory neurons.

Sensory cortex: stimulus parameter	Layers	Anesthesia	Age	Species	Inhibitory and excitatory tuning widths	References
V1: Orientation	Mixed, 2/3, 4	Thiopental, ketamine/thiopental, diprivan/sufentanil citrate, pentobarbital/chlorprothixene	Adult	Cat, Mouse	Co-tuned	Anderson et al., 2000; Martinez et al., 2002; Tan et al., 2011
	4	Urethane/chlorprothixene	Pre-critical period	Mouse	Co-tuned	Li et al., 2012b
	Mixed	Alfaxolone/alfadolone,	Mixed	Cat	Mixed	Monier et al., 2003
	5	Ketamine/thiopental, diprivan/sufentanil citrate	Adult	Cat	Different excitatory and inhibitory tuning	Martinez et al., 2002
	2/3, 4	Urethane/chlorprothixene	Adult, critical period	Mouse	Inhibition broader than excitation	Liu et al., 2011; Li et al., 2012b
A1: Frequency	Mixed, 3–5	Ketamine/medetomidine, pentobarbital	Pre-critical period, critical period, adult	Rat, Mouse	Co-tuned/Similar	Wehr and Zador, 2003; Zhang et al., 2003; Tan et al., 2004; Froemke et al., 2007
	Mixed	Ketamine/xylazine	Pre-critical period	Rat	Different excitatory and inhibitory tuning	Dorrn et al., 2010
	Mixed	Ketamine/xylazine	Adult	Rat	Co-tuned	Dorrn et al., 2010
	4	Ketamine/xylazine	Pre-critical period	Rat	Co-tuned	Sun et al., 2010
	4	Ketamine/xylazine	Adult	Rat	Inhibition broader than excitation	Wu et al., 2008; Sun et al., 2010
A1: Intensity	Mixed	Ketamine/medetomidine	Pre-critical period/critical period	Rat	Co-tuned/Similar	Wehr and Zador, 2003
	Mixed, 4	Ketamine/xylazine, pentobarbital	Adult	Rat	Umbalanced	Wu et al., 2006; Tan et al., 2007
Pir: Odor	2/3	Urethane, ketamine	Pre-critical period, critical period	Rat	Inhibition broader than excitation	Poo and Isaacson, 2009

for the direction of a whisker deflection; however, the temporal gap before the onset of inhibition is greatest for the preferred direction (Wilent and Contreras, 2005). In layer 6 of rat A1, a majority of pyramidal neurons receive strong tone-evoked synaptic inhibition prior to excitation and are thus inhibited by tonal stimulation (Zhou et al., 2010). Interestingly, the time course of excitatory and inhibitory responses to oriented gratings in V1 differs between species: inhibition and excitation wax and wane in opposition in cats (Hirsch et al., 1998; Anderson et al., 2000; Monier et al., 2003; Priebe and Ferster, 2005; Tan et al., 2011), while they are modulated synchronously in the mouse (Tan et al., 2011). Species-specific differences in light and dark receptive subfields have been proposed to underlie this discrepancy (Tan et al., 2011).

In addition to possibly mediating spike timing and tuning curves, inhibition may play a role in response suppression and in maintaining sparseness of spiking responses. Response suppression occurs when a stimulus that would not by itself alter firing of a neuron inhibits the response of that neuron to another stimulus. While GABAergic neurons mediate some forms of response suppression, they likely do not mediate all response suppression. For example, when two tones of the same frequency are played in rapid succession, the cortical response to the second tone is suppressed. As both the onset and offset of stimuli can evoke neuronal responses with transient inhibitory and excitatory components, one hypothesis explaining this effect involves the first tone's offset evoking inhibition that suppresses the response to the second tone (Borg-Graham et al., 1998; Wehr and Zador, 2005; Scholl et al., 2010). However, Scholl et al. found that in rat A1 there is different frequency tuning of inputs for tone onset and offset (Scholl et al., 2010). This differential tuning allows the onset and offset of a given tone to evoke different excitatory and inhibitory current ratios, albeit transiently. Therefore, "forward" suppression of a second tone of the same frequency can only be mediated through feedforward inhibition generated by the first tone's onset if the second tone follows within the brief time window of the first tone's onset inhibitory current (Scholl et al., 2010).

In V1, a stimulus placed adjacent to a neuron's spiking receptive field can alter its responses to stimuli within the spiking receptive field, a phenomenon referred to as surround suppression. Surround suppression has been shown to be mediated, at least in part, through intracortical inhibition (Ozeki et al., 2009; Haider et al., 2010; Adesnik et al., 2012). However, there may be multiple mechanisms through which surround suppression occurs, depending on the nature of the stimuli. For example, in cats, surround suppression by optimally oriented gratings decreases the total excitatory and inhibitory inputs onto a cell: a transient increase in inhibition can be evoked by surround stimuli, which ultimately leads to suppression of both cortical excitatory and inhibitory inputs (Ozeki et al., 2009). For "naturalistic" stimuli, movies of wildlife, adding stimuli to the receptive field surround increases spiking reliability and firing sparseness (Haider et al., 2010). This effect is mediated through an increase in evoked inhibitory potentials throughout the stimulus duration and corresponds with an increase in FS interneuron firing (Haider et al., 2010). Perhaps the discrepancies between these studies could be accounted for by the ever changing nature of the "naturalistic" stimuli, with the many moving edges evoking successive bouts of inhibition that blend together (Haider et al., 2010). In layer 2/3 of awake mice, inhibition through SOM positive interneurons accounts for approximately 30% of surround suppression by optimally oriented gratings (Adesnik et al., 2012).

In layer 5 of S1, stimulation of the ipsilateral paw just prior to stimulating the contralateral paw suppresses the neuronal response to the contralateral paw. This suppression is mediated by layer 1 interneurons driven by colossal projections that provide dendritic inhibition via $GABA_B$ receptor activation (Pérez-Garci et al., 2006; Palmer et al., 2012). These findings suggest that multiple intracortical mechanisms exist to generate suppression, with different mechanisms likely contributing to the suppression of different types of stimuli.

Inhibition is ideally suited to contribute to the maintenance of response sparseness. Interestingly, while S1/barrel cortex layer 2/3 pyramidal neurons uniformly show membrane potential responses to whisker stimulation, only ∼10% spike during active touch (Crochet et al., 2011). This has been attributed to whisker responses having reversal potentials below spike threshold (Crochet et al., 2011; Sachidhanandam et al., 2013). These responses have reversal potentials well below those of excitatory receptors, suggesting that GABA receptor mediated currents may contribute to preventing these neurons from reaching spike threshold (Sachidhanandam et al., 2013). In favor of this interpretation, there is experimental evidence that whisker stimulation activates inhibitory neurons (Simons, 1978; Simons and Carvell, 1989; Swadlow, 1989; Armstrong-James et al., 1993; Zhu and Connors, 1999; Bruno and Simons, 2002; Swadlow and Gusev, 2002; Beierlein et al., 2003; Zhu et al., 2004; Zhu and Zhu, 2004; Gabernet et al., 2005; Cruikshank et al., 2007; Kwegyir-Afful et al., 2013; Lee et al., 2013; Sachidhanandam et al., 2013) and increases inhibitory drive onto excitatory neurons (Wilent and Contreras, 2005). Optogenetic photoactivation of PV neurons during a detection task both masked subthreshold responses and prevented behavioral responses (Sachidhanandam et al., 2013). These results suggest that inhibition can be precisely tuned to allow sparse firing in layer 2/3, keeping most neurons just below spike threshold after stimulation.

Additional circuit precision can be gained through subcellular compartment specific inhibition. While S1 layer 5 pyramidal neurons are rarely connected to each other directly, they are more commonly coupled disynaptically through layer 5 Martinotti cells (Silberberg and Markram, 2007). When activated, these Martinotti cells can prevent the generation of calcium spikes in the dendritic tufts of layer 5 pyramidal neurons (Murayama et al., 2009). Modeling of this circuit suggests that Martinotti cells are responsible for maintaining the dynamic range of dendritic calcium responses to paw shock that are observed *in vivo* (Murayama et al., 2009).

GABAergic neurons do not only contact excitatory neurons, but form networks of electrically and synaptically coupled interneurons, often comprised of the same cell type, that can be activated synchronously (Hestrin and Galarreta, 2005; Oswald et al., 2009; Pfeffer et al., 2013). This connectivity is especially well suited to allow for synchronization of local circuits and is

thought to contribute to the generation of oscillatory network behaviors (Tamás et al., 2000; Whittington et al., 2000; Oswald et al., 2009). The synchronicity of membrane potential oscillations between neurons can vary depending on stimuli. Neurons with similar receptive fields increase their membrane potential cross-correlation in response to optimal stimuli and decrease it in response to non-optimal stimuli (Lampl et al., 1999). Regardless of relative tuning, coherence in the gamma band between neurons increases for a wide variety of visual stimulus conditions (Yu and Ferster, 2010). Fast rhythmic bursting cells can fire bursts of action potentials in the gamma range (20–80 Hz) in response to visual stimuli, possibly contributing to visually evoked gamma oscillations (Cardin et al., 2005). This evoked gamma activity is dependent upon synaptic drive and is not generated only by the membrane properties of the chattering cells (Cardin et al., 2005). Gamma frequency oscillations measured in local field potentials can be evoked *in vivo* by optogenetic stimulation of PV positive neurons, suggesting that FS neurons may drive stimulus-evoked activity in the gamma band (Cardin et al., 2009). Photoactivation of these neurons to drive gamma oscillations during whisker deflection increases the precision of excitatory neuron responses and alters the amplitude and timing of responses in an oscillation phase dependent manner (Cardin et al., 2009).

Future work will be necessary to unravel how specific interneuron subtypes contribute to modulating sensory responses, fully understand the roles they play in higher order processing, and address whether there are specific conditions under which lateral inhibition sharpens receptive fields. One recent study in auditory cortex has begun to examine the cell type specific contributions of inhibition to modulating pyramidal cell activity during learning: VIP neurons were found to respond preferentially to reinforcement (Pi et al., 2013). SOM neurons were rapidly inhibited by VIP neurons, which caused an increase in firing of a subset of pyramidal neurons. This finding was interpreted to mean that activation of VIP neurons by reinforcement cues alters the gain of pyramidal neurons via disinhibition of SOM neurons (Pi et al., 2013). Together, the results presented in this section raise the intriguing possibility that the diversity in morphology and biophysical properties of GABAergic neurons is matched by their many functions.

DEVELOPMENT OF GABAergic NEURONS AND SYNAPSES IN SENSORY CORTEX

GABAergic neurons and synapses undergo a process of postnatal maturation which extends for quite some time after birth and co-occurs with the development of many functional properties of excitatory neurons (Espinosa and Stryker, 2012). Here, we will focus on the developmental changes that occur after inhibitory neurons have reached their target location within the cortical mantle and after GABAergic synaptic transmission has transitioned from excitatory to inhibitory. Exhaustive readings of earlier developmental processes can be found elsewhere (see Cherubini et al., 1991; Xu et al., 2003; Li and Xu, 2008; Gelman and Marín, 2010; Rudy et al., 2011; Ben-Ari et al., 2012; Ciceri et al., 2013; Taniguchi, 2014).

The intrinsic properties of inhibitory neurons change during postnatal development. In rodent V1, PV FS neurons show a progressive decrease in membrane time constant, cell capacitance and action potential width and an increase in intrinsic excitability during the first 2 weeks after eye opening (Goldberg et al., 2011; Lazarus and Huang, 2011). Conversely, SOM positive neurons show a progressive increase in membrane time constant, input resistance and spike frequency adaptation (Lazarus and Huang, 2011). The properties of inhibitory synapses also change during this developmental time window (Morales et al., 2002; Heinen et al., 2004; Maffei and Turrigiano, 2008a; Pinto et al., 2010). During the postnatal weeks following eye opening there is a progressive increase in a1 containing $GABA_A$ receptors and a progressive decrease in a3 and a5 containing $GABA_A$ receptors. Significant changes also occur in the expression of γ, δ and ε subunits (Heinen et al., 2004). Changes in receptor subunit composition underlie changes in conductance, clustering and susceptibility to allosteric modulators: all factors that play fundamental roles in determining the amplitude and time course of postsynaptic events (Bosman et al., 2002; Heinen et al., 2004; Möhler, 2006; Eyre et al., 2012). In mature brains $GABA_A$ receptors containing specific α subunits are primarily located in specific subcellular compartments: α1 and α3 containing receptors are enriched at synapses on the soma and perisomatic regions of pyramidal neurons (Klausberger et al., 2002), α2 containing receptors are preferentially located at axo-axonal synapses (Nusser et al., 1996), α5 containing receptors are preferentially located at dendritic synapses (Ali and Thomson, 2008), and α4 and α6 containing receptors predominantly mediate extrasynaptic inhibition (Wisden et al., 2002; Chandra et al., 2006; Wu et al., 2012). Whether $GABA_A$ receptor subunits are similarly localized during development is not known; however, the observed changes in levels of expression suggest that changes in the subcellular targeting of GABAergic synapses may occur.

Comparable processes of protracted maturation of inhibitory synapses have been reported in A1 and S1. In both regions there is a progressive increase in spontaneous IPSC amplitude (Kobayashi et al., 2008; Zhang et al., 2011; Takesian et al., 2012), and in A1 there are many developmentally regulated changes in inhibitory neuron intrinsic properties and in evoked synaptic strength (Takesian et al., 2010, 2012, 2013; Oswald and Reyes, 2011; Kinnischtzke et al., 2012).

Developmental changes in inhibitory circuit function go beyond subunit composition, strength and kinetic properties. Inhibitory drive tends to increase during postnatal development (Blue and Parnavelas, 1983; Morales et al., 2002; Chattopadhyaya et al., 2004; Maffei and Turrigiano, 2008a; Kuhlman et al., 2011; Li et al., 2012b). Changes in the strength of inhibitory inputs have been shown to decrease the ability of excitatory neurons to fire action potentials (Saraga et al., 2008; Pouille et al., 2013). Thus, developmental regulation of inhibitory synaptic strength may have significant effects on the activity of excitatory neurons. Precise patterns of neural activity are required for the induction of different forms of plasticity; therefore, changes in circuit activity may alter how stimuli drive changes at synapses. Patterns of activity or sensory experiences that facilitate the induction of long term potentiation (LTPi) or depression (LTDi) at inhibitory synapses could gate the induction of specific forms of plasticity at glutamatergic synapses in cortical

circuits (Levelt and Hübener, 2012). Gating of excitatory plasticity could occur by altering activity patterns of glutamatergic neurons (Aton et al., 2013), by activating neuromodulatory signals (Huang et al., 2013) and/or through the activation of intracellular signaling cascades (Hayama et al., 2013; Wang and Maffei, 2014).

The capacity for plasticity at inhibitory synapses in V1 changes sharply during postnatal development at a time point corresponding to the transition between the pre-critical and the critical periods for visual cortical plasticity (Lefort et al., 2013). In response to the same pattern of activity, monosynaptic connections from FS neurons onto layer 4 pyramidal neurons show LTDi in the pre-critical period and LTPi in the critical period (Lefort et al., 2013). This shift in capacity for plasticity parallels that reported for layer 4 recurrent excitatory connections (Wang et al., 2012). Surprisingly, the switch in sign of plasticity at excitatory synapses can be reversed by inducing inhibitory plasticity in a connection specific fashion, indicating that inhibitory inputs may contribute to selective rewiring of local circuits despite their widespread connectivity (Wang and Maffei, 2014). This interaction between inhibitory and excitatory plasticity is mediated by G-protein signaling; therefore, it may affect the pyramidal neuron long after its induction (Wang and Maffei, 2014). Such a signaling crosstalk between mechanisms for excitatory and inhibitory plasticity could provide a memory trace that promotes experience-dependent rewiring of local microcircuits with a high degree of specificity and contributes to developmental circuit refinement.

During the developmental window in which inhibitory synapses mature, there are also substantial changes in glutamatergic receptor subunit composition, subcellular localization and function (Carmignoto and Vicini, 1992; Nase et al., 1999; Hensch, 2004; Corlew et al., 2007; Yashiro and Philpot, 2008), voltage signal propagation through the cortical circuit (Barkat et al., 2011; Griffen et al., 2013) and receptive field properties (Fagiolini et al., 1994; Katz and Shatz, 1996; Huang et al., 1999; Zhang et al., 2001; Inan and Crair, 2007; Wang et al., 2010; Espinosa and Stryker, 2012; Chen et al., 2014; Li et al., 2014a). Whether and how GABAergic neurotransmission contributes to these developmental processes is not fully understood. Evidence is beginning to emerge that changes in the receptive fields of the inhibitory drive onto excitatory neurons may at least partially underlie developmental receptive field sharpening (Dorrn et al., 2010; Sun et al., 2010; Li et al., 2012b). However, the underpinning synaptic and circuit mechanisms of this process remain elusive.

SENSORY DEPRIVATION IMPAIRS THE DEVELOPMENT OF GABAergic INHIBITION

The maturation of inhibitory synapses is strongly influenced by sensory experience. Visual deprivation induced by raising animals in the dark from birth or shortly thereafter disrupts visual response properties (Benevento et al., 1992; Fagiolini et al., 1994; Gianfranceschi et al., 2003; but see Rochefort et al., 2011) and delays the formation of mature innervation onto excitatory neurons; whereas dark rearing started after eye opening does not affect inhibitory drive (Morales et al., 2002; Maffei et al., 2010). Dark rearing decreases the level of expression of GAD65 in layer 2/3 inhibitory neurons (Kreczko et al., 2009) and prevents postnatal increases in IPSC amplitude onto pyramidal neurons from occurring, consistent with a delay in GABAergic input maturation (Morales et al., 2002). Interestingly, dark rearing mice blocks the developmental broadening of the orientation tuning of PV neurons, but it does not fully prevent the developmental sharpening of the orientation tuning of pyramidal neurons in layers 2–4 (Kuhlman et al., 2011; Rochefort et al., 2011; Li et al., 2012b), or of the inhibitory inputs to layer 4 pyramidal neurons (Li et al., 2012b). Dark rearing also delays the maturational increase in the coincidence of binocular inputs to pyramidal neurons in layers 2–4 (Chen et al., 2014). Pharmacologically enhancing inhibition with benzodiazepines early in development disrupts binocular matching of orientation preference in complex cells (Wang et al., 2010, 2013a), widens the spacing of orientation columns and disrupts direction selectivity (Hensch and Stryker, 2004). Together with sensory deprivation experiments, these results suggest that the normal development of GABAergic inhibition is essential for the proper maturation of visual cortex.

Auditory experience also plays a major role in the development of inhibitory inputs in A1, as early hearing loss, either conductive or sensorineural, alters GABAergic neurotransmission (Kotak et al., 2005, 2008; Takesian et al., 2010, 2012). Early conductive hearing loss delays the developmental decrease in IPSC decay time constant (Takesian et al., 2012), and produces a significant, long lasting decrease in spontaneous IPSC amplitude, suggesting the induction of long term depression of inhibitory drive (Takesian et al., 2012). Auditory experience is not only necessary for the maturation of inhibition in A1, but also regulates the capacity for plasticity at inhibitory synapses (Xu et al., 2010) and the feedforward thalamocortical drive onto different classes of inhibitory neurons (Takesian et al., 2013).

While whisker plucking prevents the maturation of feedforward inhibition onto excitatory neurons in the barrel cortex (Chittajallu and Isaac, 2010), the mechanisms engaged in driving GABAergic synapse maturation in S1 are unknown. Across cortices, early sensory deprivation delays the maturation of inhibitory synapses, but how experience-dependent, developmentally regulated cellular and synaptic changes in inhibition directly influence sensory responses is unclear.

GABAergic MATURATION AND CRITICAL PERIOD PLASTICITY

Experiments with early sensory deprivation have shown that sensory experience is necessary for the maturation of inhibitory neurons and their synapses. However, if sensory manipulations are started later in postnatal development, for example during identified sensitive periods whose onset has been shown to correlate with the time course of the maturation of inhibition, sensory cortex is highly sensitive to even brief alterations in sensory drive (Hubel and Wiesel, 1970; Carmignoto and Vicini, 1992; Finnerty et al., 1999; Morales et al., 2002; Hensch, 2004; Maffei and Turrigiano, 2008a; Espinosa and Stryker, 2012; Levelt and Hübener, 2012). For example, monocular lid suture decreases the ratio of V1 neuronal spiking responses to the stimulation of the closed eye compared with the open eye, a phenomenon known as ocular dominance plasticity (Wiesel and Hubel, 1963). Ocular

dominance plasticity is effectively induced by lid suture during a limited time window in postnatal development known as a critical period (Hubel and Wiesel, 1970; Fagiolini et al., 1994; Gordon and Stryker, 1996).

The maturation of inhibitory synapses has been proposed as a critical step for the modulation of the onset and duration of critical periods (Hensch et al., 1998). Manipulations of inhibition *in vivo* during early postnatal development shift the onset of the critical period for visual cortical plasticity in V1. In GAD65 knockout mice, which have low levels of GABA throughout life, the critical period never opens (Fagiolini and Hensch, 2000). Increasing intracortical inhibitory drive with benzodiazepines or zolpidem in early development, or at any time in GAD65 knockouts, leads to the onset of a window for ocular dominance plasticity (Fagiolini and Hensch, 2000; Fagiolini et al., 2004; Wang et al., 2013a; Chen et al., 2014). This effect is mediated by $GABA_A$ receptors containing α1 subunits, which are preferentially located at FS to pyramidal neuron somatic synapses (Fagiolini and Hensch, 2000; Klausberger et al., 2002; Iwai et al., 2003; Fagiolini et al., 2004; Katagiri et al., 2007). Either overexpressing BDNF or removing polysialic acid accelerates the development of inhibitory synapses and causes a precocious critical period (Hanover et al., 1999; Huang et al., 1999; Di Cristo et al., 2007). Similarly, sensory deprivation by dark rearing prolongs the onset of sensitivity to ocular dominance plasticity until the animal is introduced to light (Cynader and Mitchell, 1980; Mower and Christen, 1985; Mower, 1991; Fagiolini et al., 1994).

Conversely, pharmacologically decreasing inhibition in adult animals can reopen a window for ocular dominance plasticity (Harauzov et al., 2010; Maya-Vetencourt et al., 2012). This can be accomplished by reducing cortical GABA with GAD inhibitors, with the peptide hormone IGF-1, or by blocking $GABA_A$ receptors (Harauzov et al., 2010; Maya-Vetencourt et al., 2012). Environmental enrichment, which also decreases intracortical inhibition, can restore the capacity for ocular dominance plasticity in adult rodents in an IGF-1 dependent manner (Sale et al., 2007; Baroncelli et al., 2010; Maya-Vetencourt et al., 2012). Direct evidence that the age of inhibitory neurons, rather than overall inhibitory tone, plays a major role in critical period regulation comes from experiments where implants of GABAergic neural progenitors from embryonic mice were made into V1 of older mice. These implants later opened a window for ocular dominance plasticity that matched the age of the implanted neurons (Southwell et al., 2010). Substantial evidence exists that the maturation of inhibition regulates the timing of critical periods. Uniformly, manipulations that prevent that maturation of inhibition prevent the onset of critical periods, while those that enhance inhibition early in development cause a precocious critical period. Once inhibition has matured, reducing inhibition acutely can restore the capacity for ocular dominance plasticity. Manipulations that delay or accelerate the onset of the critical period for visual cortical plasticity, dark rearing and benzodiazepine application, also disrupt receptive field properties (see previous section), suggesting that inhibitory neurons play a fundamental role in the development of sensory cortex under normal conditions.

SENSORY ALTERATION INDUCED PLASTICITY AT INHIBITORY SYNAPSES

Alterations in sensory experience are not only dependent upon cortical inhibitory tone to induce plasticity; they also induce specific plastic changes at inhibitory synapses. Altering sensory drive engages inhibitory plasticity differently depending on the age of the animal, the nature of the change in sensory drive, and the specific synapses examined. For example, monocular lid suture induces rapid changes in inhibitory synaptic strength and drive, whether the lid is sutured before or after eye opening (Maffei et al., 2004, 2006, 2010; Wang et al., 2011; Kuhlman et al., 2013; Wang and Maffei, 2014). However, brief monocular lid suture before eye opening decreases drive from FS neurons onto pyramidal neurons in layer 4 of V1 (Maffei et al., 2004), whereas lid suture of the same duration started during the critical period potentiates FS to pyramidal neuron synapses in layer 4, both pre- and post-synaptically (Maffei et al., 2006; Nahmani and Turrigiano, 2014).

Removing a single row of whiskers during the first 2–3 postnatal weeks leads to an early potentiation of whisker responses in layer 2/3 of S1 after 3 days of deprivation followed by a loss of responses after 5–10 days (Drew and Feldman, 2009; Li et al., 2014a). Responses in layer 4 are not changed by this manipulation (Drew and Feldman, 2009; Li et al., 2014a). Different from lid suture, removing a single row of whiskers induces potentiation of FS to pyramidal neuron synapses in layer 2/3, where sensory plasticity is expressed (House et al., 2011).

In adult animals one of the main effects of sensory manipulation is the induction of structural changes in inhibitory neurons. A reduced number of dendritic spines has been reported in a subpopulation of inhibitory neurons following adult monocular retinal lesioning (Keck et al., 2011), suggesting that they may become less effectively activated by sensory stimuli. In addition, monocular lid suture in adult animals leads to a decrease in the number of inhibitory inputs onto layer 2/3 pyramidal neurons (van Versendaal et al., 2012), and the fraction of dynamic inhibitory synapses onto layer 2/3 pyramidal neuron spines and shafts increases (Chen et al., 2012). Thus, in adult V1 unilateral manipulation of visual drive induces a rapid restructuring of inhibitory inputs, possibly leading to an overall decreased inhibitory drive onto layer 2/3 pyramidal neurons.

While pharmacologically manipulating inhibition can change the potential for experience-dependent plasticity, global changes in circuit excitability may not be the ideal mechanism for fine-scale refinement of cortical circuit connectivity. Fine scale circuit refinement may require local, possibly synapse specific, regulation of inhibition. The age, layer and cell type specific changes in inhibitory synapses observed following monocular lid suture could provide highly specific modulation of inhibition. The layer 4 FS to pyramidal neuron synapses that are potentiated rapidly following monocular lid suture are sensitive to benzodiazepines, suggesting that they could play a role in critical period regulation (Maffei et al., 2006; Katagiri et al., 2007; Wang and Maffei, 2014). Induction of LTPi at these synapses, either through lid suture or acutely, switches the sign of plasticity induced at pyramidal neuron recurrent synapses by slow wave stimulation from long term potentiation of excitation (LTPe) to long term depression of

excitation (LTDe) (Wang et al., 2012; Wang and Maffei, 2014). Acute depotentiation of the FS to pyramidal neuron synapses following lid suture restores the capacity for induction of LTPe, demonstrating that the control of inhibitory plasticity over the capacity for plasticity at excitatory synapses is modulated by experience (Wang and Maffei, 2014). While synapse specific, experience-dependent inhibitory control over excitatory plasticity has only been shown to exist in one microcircuit, this finding raises the intriguing possibility that plasticity at inhibitory synapses may control the expression of other forms of plasticity throughout cortex.

Experience-dependent reductions in inhibitory drive have been proposed to have a homeostatic role in contributing to the maintenance of neuronal activity in the face of altered sensory drive (Maffei et al., 2004; Maffei and Turrigiano, 2008b; Yang et al., 2011; Shao et al., 2013). Lid suture prior to the critical period decreases inhibitory synaptic strength onto excitatory neurons in layer 4 (Maffei et al., 2004). This homeostatic plasticity could be detected as a decrease in the spontaneous inhibitory drive and an increase in the spontaneous excitatory drive onto pyramidal neurons (Maffei et al., 2004). The induction of homeostatic inhibitory plasticity by monocular lid suture is developmentally regulated and shows layer specificity (Maffei et al., 2004; Maffei and Turrigiano, 2008b).

High decibel, high frequency sounds can lead to selective high frequency hearing loss, reorganization of the frequency map in A1 and tinnitus (Yang et al., 2011). Selective hearing loss leads to decreases in spontaneous inhibitory events and tonic inhibition thought to compensate for decreased sensory drive (Yang et al., 2011). Pharmacologically enhancing inhibition successfully eliminates tinnitus, while directly reducing excitation does not (Yang et al., 2011).

Whisker removal during a critical period for S1 plasticity reduces feedforward excitatory drive from layer 4 onto both layer 2/3 FS and pyramidal neurons, likely decreasing the activation of layer 2/3 by sensory input (House et al., 2011; Li et al., 2014a). This reduction in feedforward drive is compensated for by a robust decrease in the ability of layer 2/3 pyramidal neurons to recruit recurrent inhibition (Shao et al., 2013). These results coupled with the finding of FS to pyramidal neuron synaptic potentiation in layer 2/3 (House et al., 2011) suggest that a combination of homeostatic and Hebbian plasticity mechanisms contribute to rewiring the superficial layers of the barrel cortex.

Different forms of inhibitory synaptic plasticity can be engaged to refine or control local circuits. Despite widespread connectivity (Fino and Yuste, 2011; Packer and Yuste, 2011), the diversity of inhibitory neurons, and the selective sensitivity of distinct inhibitory synapses make them well suited to contribute to the maintenance of network excitability, large scale regulation of sensory cortical circuits and fine scale experience-dependent refinement of local microcircuits.

INHIBITION AND CHANGES IN RESPONSES FOLLOWING SENSORY ALTERATIONS

Understanding how changes in GABAergic neurons and their synapses contribute to changing sensory responses and maintaining homeostasis of firing rates in the face of altered sensory drive will be essential to pushing the field of experience-dependent plasticity forward. Recent work has shown that a brief unilateral reduction in visual drive strongly modulates the binocularity of FS inhibitory neurons. While FS inhibitory neurons are normally more binocular than pyramidal neurons (Swadlow, 1988; Yazaki-Sugiyama et al., 2009; Aton et al., 2013), soon after the onset of monocular lid suture or eye patching, layer 2/3 FS neurons are more strongly driven by the closed eye (Yazaki-Sugiyama et al., 2009; Aton et al., 2013; but see Kuhlman et al., 2013), although their spontaneous firing frequency is reduced (Yazaki-Sugiyama et al., 2009; Aton et al., 2013; Hengen et al., 2013). This increase in FS responsiveness to the deprived eye could contribute to reducing excitatory neuron responsiveness to that eye (Yazaki-Sugiyama et al., 2009). Interestingly, if the unilateral deprivation is maintained for several days both changes in ocular dominance of layer 2/3 FS neurons (Yazaki-Sugiyama et al., 2009; Aton et al., 2013; Kuhlman et al., 2013) and their spontaneous firing frequency are reversed (Aton et al., 2013; Hengen et al., 2013; but see Yazaki-Sugiyama et al., 2009). In layer 4 pyramidal neurons of mice, brief monocular lid suture similarly decreases both peak evoked excitatory and inhibitory conductances, and longer deprivation selectively reduces inhibition driven by the open eye (Ma et al., 2013). Differently, in rats longer monocular lid suture reduces excitation and inhibition driven by the deprived eye equally (Iurilli et al., 2013). Together, these results suggest that other mechanisms, such as changes in thalamocortical drive (Shatz and Stryker, 1978; LeVay et al., 1980; Khibnik et al., 2010; Wang et al., 2013b) may cooperate with altered intracortical inhibition to mediate the functional effects of sensory deprivation.

At first glance, studies showing that monocular lid suture does not affect the ratio of excitatory and inhibitory inputs to layer 4 pyramidal neurons (Iurilli et al., 2013; Ma et al., 2013) appear contradictory to the hypothesis that potentiation of inhibition directly suppresses visual responses (Maffei et al., 2006). However, many questions still exist. Visual stimuli used in these experiments were either moving bars or drifting gratings presented over seconds, or noise stimuli presented for several hundred milliseconds. Stimuli were presented only to one eye at a time. Additionally, only peak responses obtained during stimuli presented at peak orientation were examined (Iurilli et al., 2013; Ma et al., 2013). However, FS neurons in visual cortex have response properties that make them ideally suited to modulate extremely rapid visual responses to stimuli presented binocularly (Swadlow and Weyand, 1987; Swadlow, 1988; Yazaki-Sugiyama et al., 2009; Kameyama et al., 2010; Cardin, 2012; Aton et al., 2013; Zhuang et al., 2013). Additionally, their broad tuning raises the possibility that they could contribute to changes in orientation tuning or response suppression following lid suture. Alternatively, changes in patterns of FS neuron activity and synaptic strength could gate activity at other neurons' synapses (Levelt and Hübener, 2012; Wang and Maffei, 2014). Finally, recordings of somatic inhibitory conductances are unable to resolve differential contributions from interneuron subgroup: changes in input from one group may be offset by opposite changes in input from other groups. A recent study examining changes in excitatory and inhibitory conductances following whisker removal underscores the difficulty in

interpreting results using this technique (Li et al., 2014a). Short and long durations of whisker trimming reduce evoked excitatory and inhibitory drive, but both decrease inhibition more than excitation. Interestingly, brief whisker trimming increases evoked spiking responses, while longer removal decreases responses (Li et al., 2014a). Thus, additional circuit mechanisms beyond evoked somatic excitatory and inhibitory balance must contribute to this change in responsiveness.

Two particularly exciting studies have shed light on possible roles of inhibitory plasticity in modulating sensory responses. In addition to reducing inhibition in layer 2/3, whisker trimming significantly reduces gamma oscillations in the circuit driven by the deprived whisker (Shao et al., 2013), suggesting that sensory changes in inhibitory drive may modulate network state. Whether reducing evoked gamma oscillations *in vivo* favors the induction of plasticity is not known. Like lid suture, long term monocular strabismus disrupts ocular dominance (Hubel and Wiesel, 1965), and strabismus leads to binocular suppression, whereby monocular stimuli elicit stronger responses than binocular stimuli (Sengpiel and Blakemore, 1994; Sengpiel et al., 1994). Strabismus was shown to induce binocular suppression through a selective increase in inhibitory drive during binocular stimulation (Scholl et al., 2013). These results suggest that changes in cortical inhibition induced by sensory experience may alter sensory processing in more subtle and complex ways than simply repressing unilateral deprived organ (whisker or eye) responses to optimal stimuli.

Changes in inhibitory drive are certainly not the sole mechanism at play in the experience-dependent rewiring of sensory cortex, as trophic factors, excitatory synapses, neuron intrinsic properties and extracellular matrix interactions have all been shown to contribute to the altered visual response properties and ocular dominance (Berardi et al., 2003; Levelt and Hübener, 2012). Determining how inhibitory neurons and their plasticity contribute to cortical function will require a more comprehensive understanding of how specific subclasses of inhibitory neurons contribute to sensory responses. Techniques that isolate the specific contribution of interneuron groups will need to be combined with detailed analyses of circuit and cellular properties. Many different mechanisms of GABAergic plasticity have been identified (see Castillo et al., 2011; Maffei, 2011). To manipulate these specific forms of inhibitory plasticity, it will be necessary to first identify the parameter space for their induction and expression. Specific mechanisms can then be manipulated to assess their roles in modulating neurons' response properties.

CONCLUSIONS

The maturation and plasticity of GABAergic neurons play fundamental roles in the development of sensory cortex. Inhibitory synaptic transmission and plasticity may affect critical period timing, specific receptive field properties, spike timing and other temporal aspects of coding, gain, neural synchrony, spontaneous and evoked firing rates, the capacity for plasticity at other synapses, and contribute to the homeostasis of circuit excitability. The role of all the distinct population of inhibitory neurons in the experience-dependent reorganization of cortical circuits has not been fully explored, but there is sufficient evidence to show that different inhibitory neuron types may be engaged to modulate different aspects of neuron response properties. The challenge ahead lies in identifying the distinct roles of inhibitory neurons in cortical circuits and in determining how their plasticity contributes to modulating other neurons' response properties, connectivity, and capacity for plasticity. Development of new tools and experimental approaches to selectively manipulate specific mechanisms of inhibitory plasticity will be instrumental for determining their role in sensory cortex development and function.

ACKNOWLEDGMENTS

Support: NIH/R01 EY019885 to Arianna Maffei. Trevor C Griffen was supported by NIHGM MSTP Training award T32-GM008444. We thank Melissa Haley for insightful comments on the manuscript.

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

Received: 20 January 2014; accepted: 12 March 2014; published online: 26 March 2014.
*Citation: Griffen TC and Maffei A (2014) GABAergic synapses: their plasticity and role in sensory cortex. Front. Cell. Neurosci. **8**:91. doi: 10.3389/fncel.2014.00091*
This article was submitted to the journal Frontiers in Cellular Neuroscience.

frontiers in
CELLULAR NEUROSCIENCE

REVIEW ARTICLE
published: 18 February 2014
doi: 10.3389/fncel.2014.00042

Regulation and functional roles of rebound potentiation at cerebellar stellate cell—Purkinje cell synapses

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Purkinje cells receive both excitatory and inhibitory synaptic inputs and send sole output from the cerebellar cortex. Long-term depression (LTD), a type of synaptic plasticity, at excitatory parallel fiber–Purkinje cell synapses has been studied extensively as a primary cellular mechanism of motor learning. On the other hand, at inhibitory synapses on a Purkinje cell, postsynaptic depolarization induces long-lasting potentiation of GABAergic synaptic transmission. This synaptic plasticity is called rebound potentiation (RP), and its molecular regulatory mechanisms have been studied. The increase in intracellular Ca^{2+} concentration caused by depolarization induces RP through enhancement of $GABA_A$ receptor ($GABA_AR$) responsiveness. RP induction depends on binding of $GABA_AR$ with $GABA_AR$ associated protein (GABARAP) which is regulated by Ca^{2+}/calmodulin-dependent kinase II (CaMKII). Whether RP is induced or not is determined by the balance between phosphorylation and de-phosphorylation activities regulated by intracellular Ca^{2+} and by metabotropic GABA and glutamate receptors. Recent studies have revealed that the subunit composition of CaMKII has significant impact on RP induction. A Purkinje cell expresses both α- and β-CaMKII, and the latter has much higher affinity for Ca^{2+}/calmodulin than the former. It was shown that when the relative amount of α- to β-CaMKII is large, RP induction is suppressed. The functional significance of RP has also been studied using transgenic mice in which a peptide inhibiting association of GABARAP and $GABA_AR$ is expressed selectively in Purkinje cells. The transgenic mice show abrogation of RP and subnormal adaptation of vestibulo-ocular reflex (VOR), a type of motor learning. Thus, RP is involved in a certain type of motor learning.

Keywords: cerebellum, Purkinje cell, synaptic plasticity, rebound potentiation, long-term potentiation, motor learning, inhibitory synapse, GABA

INTRODUCTION

The cerebellum consists of cortex and nuclei, and is involved in motor control (**Figure 1**; Ito, 1984, 2011; Llinás et al., 2004). There are two major inputs to the cerebellum, mossy fibers and climbing fibers. Mossy fibers coming from pons, medulla oblongata etc., innervate neurons in cerebellar nuclei and granule cells in the granular layer of cortex. Granule cells extend axons to the molecular layer, where they bifurcate. The bifurcated granule cell axons are called parallel fibers, and form excitatory glutamatergic synapses on dendrites of Purkinje cells and inhibitory GABAergic interneurons in the molecular layer, stellate and basket cells. Climbing fibers coming from inferior olivary nuclei innervate neurons in cerebellar nuclei and Purkinje cells. A single climbing fiber forms hundreds synapses on a Purkinje cell, and thus sends a powerful excitatory drive. Purkinje cells are GABAergic neuron, and send sole output from the cortex to nuclear neurons.

Climbing fibers are thought to code error signals (Maekawa and Simpson, 1973), and regulate activities of Purkinje cells. Activation of parallel fibers followed by activation of a climbing fiber depresses the efficacy of synaptic transmission between the activated parallel fibers and a Purkinje cell long-term. This synaptic plasticity is called long-term depression (LTD), and has been considered to be a cellular basis of motor learning such as adaptation of reflex eye movements and classical conditioning of eye blink response (Ito, 1982, 2011; du Lac et al., 1995; Thompson, 2005; Hirano, 2013a). However, mice defective in LTD were shown to display normal motor learning (Welsh et al., 2005; Schonewille et al., 2011), and the involvement of other plasticity mechanisms in motor learning has been suggested (Hansel et al., 2001; Jörntell and Hansel, 2006; Dean et al., 2010; Jörntell et al., 2010; Gao et al., 2012; Hirano, 2013a).

Plasticity also takes place at synapses other than parallel fiber-Purkinje cell synapses in the cerebellum such as excitatory synapses on granule cells, those between parallel fibers and inhibitory interneuron and those in the nuclei (Jörntell and Ekerot, 2002, 2003; D'Angelo et al., 2005; Pugh and Raman, 2006). At GABAergic synapses formed by stellate cells on Purkinje cells, three types of plasticity induced by postsynaptic depolarization have been reported (**Figure 2**), namely, depolarization-induced suppression of inhibition (DSI), depolarization-induced

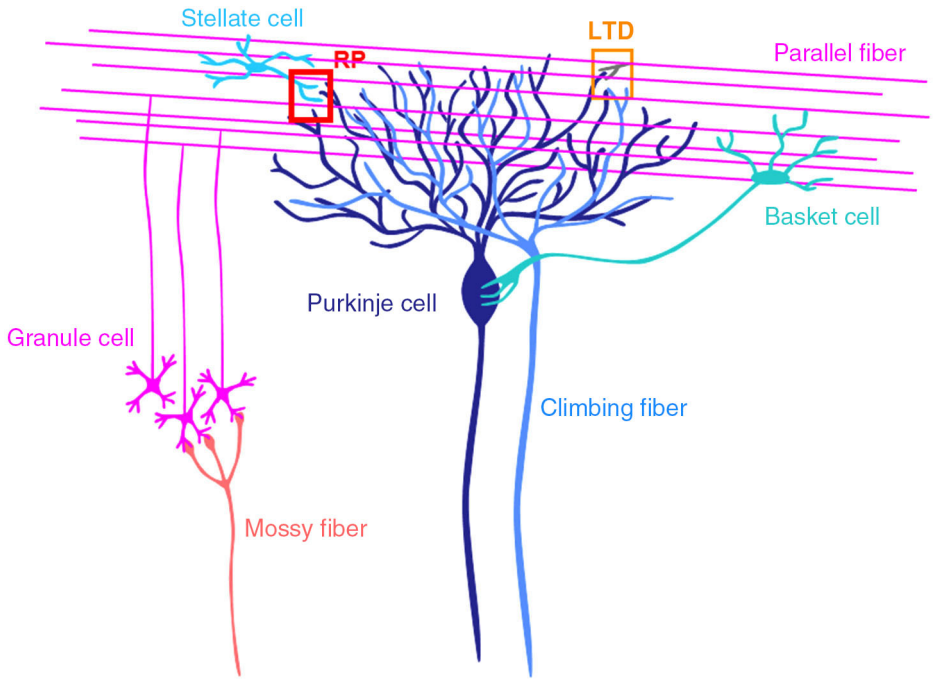

FIGURE 1 | Cerebellar cortical neuronal circuits. Mossy fibers from pontine nuclei etc., send excitatory synaptic outputs to granule cells. A granule cell forms one or a few excitatory glutamatergic synapses on a Purkinje cell, where LTD occurs depending on the activity of the granule cell and a climbing fiber. Molecular layer interneurons (stellate and basket cells) receive excitatory synaptic inputs from granule cells and inhibit Purkinje cells. At inhibitory GABAergic synapses between a stellate cell and a Purkinje cell, rebound potentiation (RP) is induced by climbing fiber activity.

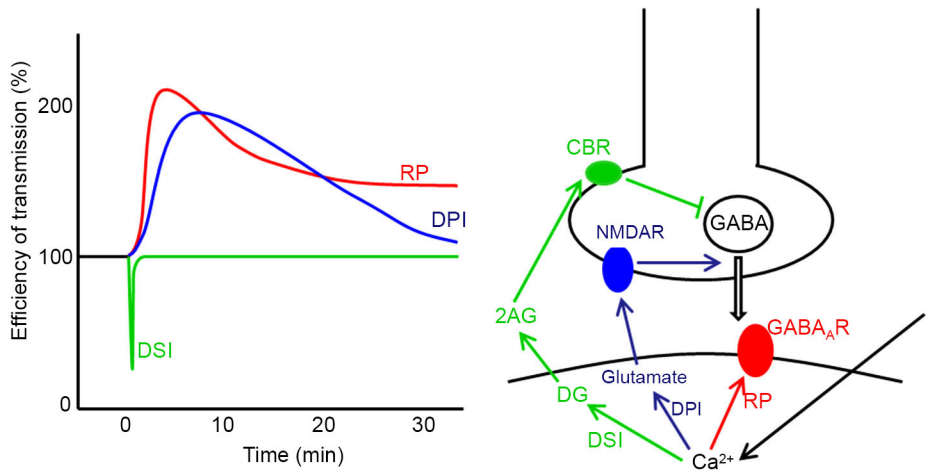

FIGURE 2 | Three forms of synaptic plasticity at stellate cell—Purkinje cell synapses. Time courses (left) and induction mechanisms (right) of DSI, DPI and RP are presented. In DSI the Ca^{2+} increase caused by postsynaptic depolarization produces diacylglycerol (DG), which is broken down to 2-arachidonylglycerol (2AG). 2AG reaches the presynaptic terminal and activates cannabinoid receptor 1 (CBR) on the cell membrane, which suppresses presynaptic vesicular release of GABA. In DPI, the intracellular Ca^{2+} increase causes postsynaptic release of glutamate, which activates presynaptic NMDA receptor (NMDAR) potentiating presynaptic GABA release. In RP, the postsynaptic Ca^{2+} increase potentiates postsynaptic $GABA_AR$ responsiveness.

potentiation of inhibition (DPI) and rebound potentiation (RP) (Hirano, 2013b). DSI is short-lasting suppression of presynaptic GABA release mediated by endocannabinoid, which is released from a Purkinje cell and binds to presynaptic cannabinoid receptor (Llano et al., 1991; Yoshida et al., 2002). DPI is longer-lasting potentiation of presynaptic GABA release mediated by glutamate, which is released from a postsynaptic Purkinje cell and binds to presynaptic NMDA receptors (Duguid and Smart, 2004).

RP occurs postsynaptically and lasts longer (Kano et al., 1992; Kawaguchi and Hirano, 2000; Tanaka et al., 2013). In RP, postsynaptic responsiveness to GABA is enhanced. These plasticity mechanisms are triggered by the postsynaptic Purkinje cell depolarization and subsequent intracellular Ca^{2+} increase (**Figure 2**). Thus, they are hetero-synaptic plasticity induced by excitatory inputs. In this article, molecular regulatory mechanisms of RP induction and functional roles of RP are reviewed.

MECHANISM OF REBOUND POTENTIATION (RP) INDUCTION

RP is induced by activation of a climbing fiber or direct depolarization of a postsynaptic Purkinje cell that causes large increase in the intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ (Kano et al., 1992; Miyakawa et al., 1992). Stimulation of a climbing fiber five times at 0.5 Hz induces RP in juvenile cerebellar slice preparations (Kano et al., 1992). However, in that study an intracellular solution containing high concentration of Cs^+ was used, and subsequent studies used direct depolarization of a Purkinje cell (Kano et al., 1992; Kawaguchi and Hirano, 2000, 2002, 2007; Kitagawa et al., 2009; Tanaka et al., 2013). Thus, patterns of climbing fiber activity sufficient to induce RP *in vivo* remain unclear. The time integral of $[Ca^{2+}]_i$ is correlated with the induction of RP, and RP is induced in an all-or-none fashion with a certain threshold (Kitagawa et al., 2009; Kawaguchi et al., 2011). RP has been monitored with the amplitude of inhibitory postsynaptic current or that of Cl^- current induced by GABA applied to dendrites, and it has been shown that RP is expressed as enhanced postsynaptic responsiveness to GABA (Kano et al., 1992; Kawaguchi and Hirano, 2000, 2002, 2007). Stellate cells form inhibitory synapses on dendrites, whereas basket cells form them on the soma of a Purkinje cell. RP has been studied primarily at stellate cell—Purkinje cell synapses in dendrites. Whether RP occurs similarly at basket cell—Purkinje cell synapses is unclear. It was difficult to record RP when GABA was applied to a soma (our unpublished observation). However, this difficulty might have been ascribed to washout of intracellular molecules necessary for RP induction caused inadvertently by the whole-cell recording conditions.

Increased intracellular Ca^{2+} binds to calmodulin, which in turn binds to Ca^{2+}/calmodulin-dependent kinase II (CaMKII). CaMKII activity is necessary for RP induction (Kano et al., 1996; Kitagawa et al., 2009). CaMKII is known to phosphorylate many proteins including $GABA_AR$ β and γ2 subunits (Moss and Smart, 1996; Brandon et al., 2002; Houston et al., 2009). Purkinje cells express α1, β2, β3 and γ2 subunits which form a heteropentameric $GABA_AR$, and β2 is more abundant than β3 (Laurie et al., 1992; Wisden et al., 1996; Pirker et al., 2000; Hirano, 2013b). Houston et al. (2008) reported the CaMKII mediated increase in IPSC amplitudes in cerebellar granule cells expressing $GABA_AR$ containing β2 subunit. Thus, direct phosphorylation of β2 subunit of $GABA_AR$ by CaMKII could be involved in RP. However, it was also reported that CaMKII potentiates α1β3γ2 $GABA_AR$ but not α1β2γ2 receptor in undifferentiated NG108-15 neuroblastoma cells (Houston and Smart, 2006), suggesting that the potentiation of β2 subunit-containing $GABA_AR$ by CaMKII may not work in some conditions or in certain cells (Houston et al., 2009). Thus, roles of direct phosphorylation of $GABA_AR$ by CaMKII in RP remain enigmatic.

Another target molecule of CaMKII in RP induction is $GABA_AR$ associated protein (GABARAP). GABARAP has a binding site for $GABA_AR$ γ2 subunit (Wang et al., 1999). RP induction is impaired by competitive inhibition of association between GABARAP and $GABA_AR$ γ2 subunit with a peptide (γ2 peptide) corresponding to the intracellular region of γ2 subunit that mediates the binding to GABARAP (Kawaguchi and Hirano, 2007). Application of this peptide after establishment of RP also attenuates once-established RP, suggesting that the interaction of GABARAP and γ2 subunit is required not only for induction of RP but also for its maintenance. Fluorescence resonance energy transfer (FRET) imaging experiments showed that GABARAP undergoes a sustained structural change in response to depolarization of a Purkinje cell (Kawaguchi and Hirano, 2007). This conformational change of GABARAP depends on activity of CaMKII. Further, single amino acid replacement of GABARAP V33E blocks structural change of GABARAP and suppresses RP induction. Thus, CaMKII-mediated conformational change of GABARAP seems to be essential for RP. GABARAP is involved in intracellular trafficking and targeting of $GABA_AR$ to the cell membrane (Kneussel et al., 2000; Kittler et al., 2001; Moss and Smart, 2001; Kneussel, 2002; Nymann-Andersen et al., 2002; Leil et al., 2004; Lüscher and Keller, 2004; Chen and Olsen, 2007; Kanematsu et al., 2007). Thus, GABARAP might induce RP through facilitating $GABA_AR$ transport to the cell membrane. In hippocampal neurons, inhibitory synaptic potentiation is induced by activation of NMDA-type glutamate receptors through GABARAP-dependent exocytosis of $GABA_AR$ (Marsden et al., 2007). Another possible role of GABARAP in RP is to enhance the function of individual $GABA_AR$ by increasing the single channel conductance or the open time (Everitt et al., 2004; Luu et al., 2006). GABARAP is also known to bind to tubulin, and it has been suggested that association of GABARAP with tubulin is required for RP induction (Kawaguchi and Hirano, 2007).

MECHANISM OF REBOUND POTENTIATION (RP) SUPPRESSION

RP is induced by cell-wide depolarization of a Purkinje cell caused by hetero-synaptic excitatory climbing fiber inputs (Kano et al., 1992). Thus, RP should occur at many inhibitory synapses on a Purkinje cell simultaneously, and should not be synapse-specific. However, there is a synapse-specific regulatory mechanism for RP induction. GABAergic synaptic transmission or $GABA_B$ receptor activation during the postsynaptic depolarization suppresses RP (Kawaguchi and Hirano, 2000). This regulation is unique in that homo-synaptic activity suppresses induction of synaptic plasticity. Usually, homo-synaptic activity triggers the plasticity of transmission.

This $GABA_B$ receptor-dependent suppression of synaptic plasticity is mediated by down-regulation of the activity of protein kinase A (PKA). It was revealed that down-regulation of PKA activity decreases the amount of phosphorylated dopamine- and cyclic adenosine monophosphate (cAMP)-regulated phosphoprotein 32 kDa (DARPP-32; Kawaguchi and Hirano, 2002; **Figure 3**). Phosphorylated DARPP-32 is known to inhibit protein phosphatase 1 (PP1), which de-phosphorylates CaMKII and

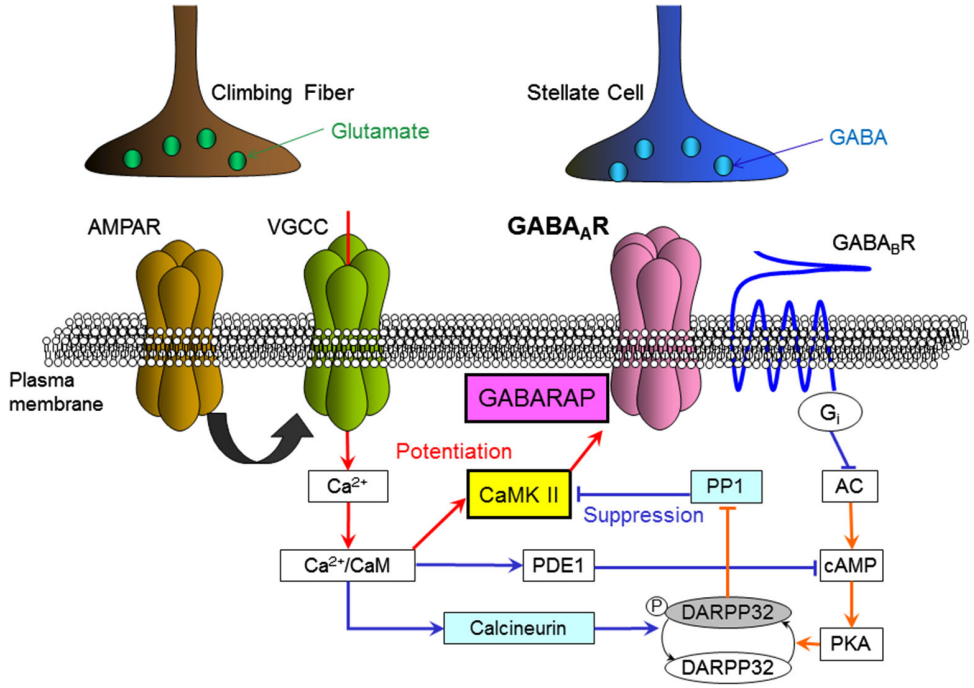

FIGURE 3 | Intracellular molecular signaling cascades regulating RP. Arrows indicate an increase, activation or enhancement, and T-bars indicate a decrease or suppression. Red lines indicate signal transmissions which work to induce RP, and blue lines indicate those work to suppress RP. AMPAR, AMPA-type glutamate receptor; VGCC, voltage-gated Ca^{2+} channel; $GABA_AR$, $GABA_A$ receptor; $GABA_BR$, $GABA_B$ receptor; CaM, calmodulin; CaMKII, Ca^{2+}/calmodulin-dependent kinase II; GABARAP, $GABA_AR$ associated protein; PDE1, phosphodiesterase 1, PP1, protein phosphatase 1; DARPP32, dopamine and cAMP-regulated phospho-protein 32 kDa; PKA, protein kinase A; cAMP, cyclic-adenosine-monophosphate; AC, adenylyl cyclase; Gi, Gi protein.

other phosphorylated proteins (Greengard et al., 1999). Thus, $GABA_B$ receptor activation works to enhance PP1 activity counteracting CaMKII. It was also shown that a Ca^{2+}-dependent phosphatase calcineurin de-phosphorylates DARPP-32 upon a $[Ca^{2+}]_i$ increase and supports suppression of RP (Kawaguchi and Hirano, 2002). A later study showed that the basal PKA activity in a Purkinje cell is partly supported by the activity of metabotropic glutamate receptor mGluR1 (Sugiyama et al., 2008).

SIGNALING CASCADE REGULATING REBOUND POTENTIATION (RP)

The preceding sections have introduced molecules involved in regulation of RP. Among them CaMKII is a key molecule for RP induction. There are two subtypes of CaMKII, α and β, and the relative expression level of β-CaMKII to α-CaMKII is higher in the cerebellum than in the forebrain (McGuinness et al., 1985; Walaas et al., 1988). In the cerebellar cortex β-CaMKII is expressed in several types of cells including Purkinje cells, whereas α-CaMKII is expressed only in Purkinje cells. It has been reported that the relative amounts of α- and β-CaMKII change depending on the neuronal activity and developmental stage in the mammalian central nervous system (Bayer et al., 1999; Thiagarajan et al., 2002). β-CaMKII has much higher affinity to Ca^{2+}/calmodulin than α-CaMKII (Brocke et al., 1999). In addition β-CaMKII binds to actin but α-CaMKII does not (Okamoto et al., 2009). Thus, subtypes of CaMKII may have different roles in a Purkinje cell. Recently, we addressed this point by overexpressing or knocking-down each type of CaMKII, and found that the subunit composition of CaMKII has a significant impact on RP induction (Nagasaki et al., 2012). Suppression of the expression of β-CaMKII but not that of α-CaMKII inhibits RP induction, whereas overexpression of α-CaMKII but not that of β-CaMKII inhibits the induction. Thus, the relative amount of β- to α-CaMKII seems to be critical for RP induction.

Interactions among molecules regulating RP including CaMKII are complex, as there are multiple branchings and feedback loops in the signaling cascades (**Figure 3**). Thus, it is difficult to intuitively predict how they behave quantitatively. To address this question, a theoretical model of molecular signaling networks for RP regulation has been built and computational simulation has been performed (Kitagawa et al., 2009; Kawaguchi et al., 2011). During this process phosphodiesterase 1 (PDE1), a Ca^{2+}/calmodulin-dependent enzyme that breaks down cAMP, was added as a critical element. The simulation reproduced essential features of induction and suppression of RP, and suggested that PDE1 plays a predominant role in determination of the Ca^{2+} threshold for RP induction (Kitagawa et al., 2009). Regulation of RP induction by a cell adhesion molecule integrin was also reported (Kawaguchi and Hirano, 2006).

Ca^{2+} CONTEXT REGULATES REBOUND POTENTIATION (RP)

RP induction depends on leaky integration of the intracellular Ca^{2+} concentration (Kawaguchi et al., 2011) as induction of LTD at glutamatergic parallel fiber—Purkinje cell synapses does

(Tanaka et al., 2007). However, it is not just integration of the Ca^{2+} signal that is critical for RP induction. We found that the context or order of the Ca^{2+} signal affects RP induction (Kawaguchi et al., 2011). Either a large and short increase in the intracellular Ca^{2+} concentration, or a small and long one can induce RP by itself. However, when a large and short increase is followed by a small and long increase, RP is not induced. In contrast, when the order is reversed, RP is induced. Thus, RP induction depends on the context or the time course of intracellular Ca^{2+} change. It was suggested that this interesting context-dependence of RP induction on the intracellular Ca^{2+} concentration is brought about by context-dependent autophosphorylation at Thr305/306 of CaMKII, which negatively regulates the subsequent Ca^{2+}/calmodulin-dependent activation of CaMKII (**Figure 3**).

INVOLVEMENT OF REBOUND POTENTIATION (RP) IN MOTOR LEARNING

Until recently there was no experimental evidence about roles of RP in cerebellar functions. We thought that RP might work together with LTD for establishment of motor learning, because activation of an inferior olivary neuron contributes to induction of both LTD and RP (Kano et al., 1992; Ito, 2011; Hirano, 2013a), and also because both down-regulation of excitatory synaptic inputs by LTD and up-regulation of inhibitory synaptic inputs by RP should work to suppress activity of a Purkinje cell. To test this idea, we generated transgenic mice defective in RP (Tanaka et al., 2013). As explained above, binding of $GABA_AR$ and an intracellular protein GABARAP is necessary for RP induction, and γ2 peptide which blocks this binding suppresses the induction. Transgenic mice which express γ2 peptide fused to a fluorescent protein only in Purkinje cells were generated. The transgenic mice do not show RP as we expected, and other physiological and morphological properties of the cerebellum including LTD induction appear normal.

Then, we evaluated the motor control and learning ability of the transgenic mice by examining reflex eye movement, vestibulo-ocular reflex (VOR; **Figure 4**). VOR is a reflex to turn an eyeball in the opposite direction of head turn, and works to stabilize visual image during head motion (Robinson, 1981). VOR undergoes adaptive modification in the direction to reduce image slip on a retina, which has been regarded as a model paradigm of cerebellum-dependent motor learning (Ito, 1982, 2011; Nagao, 1989; Lisberger et al., 1994; du Lac et al., 1995; Hirata and Highstein, 2001; Katoh et al., 2005; Hirano, 2013a). In experiments, a mouse is rotated sinusoidally on a rotating table, and a surrounding external screen with vertical black and white stripes is also rotated simultaneously (Tanaka et al., 2013). When the screen rotation is in the opposite direction to mouse rotation, the gain of VOR increases gradually in a wild-type mouse, and when the rotation is in the same direction, the gain decreases. These changes of VOR in a wild-type mouse are in the direction to reduce image motion on a retina and adaptive (**Figure 4**). These adaptive modifications of VOR amplitudes are suppressed in the transgenic

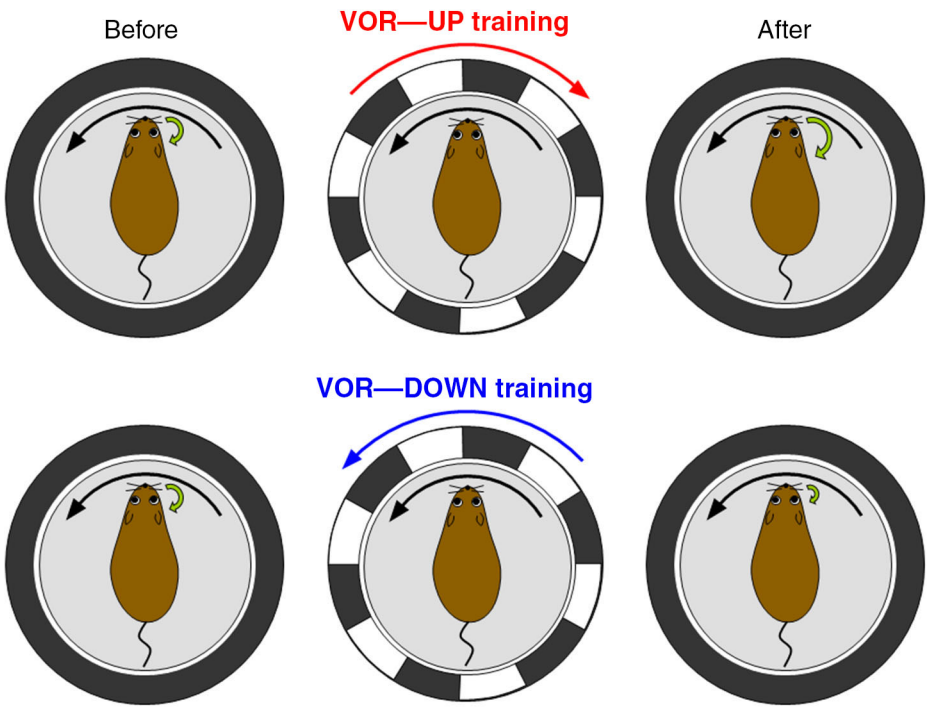

FIGURE 4 | Vestibulo-ocular reflex (VOR) and its adaptation. VOR is induced by rotating a turntable on which a mouse is fixed in the dark. In VOR eyeballs turn in the opposite direction of head turn. VOR undergoes adaptive modifications. When a wild-type mouse and a surrounding screen with vertical black and white stripes are rotated in opposite directions in the light (VOR-up training), the gain of VOR increases gradually. In contrast, when the rotations are in the same direction (VOR-down training), the gain decreases.

mice defective in RP. Thus, transgenic mice defective in RP show defects in a type of motor learning, indicating that RP contributes to motor learning. However, it should be noted that these results do not rule out a possible contribution of LTD or other plasticity to motor learning. Indeed, adaptation of optokinetic response, another type of reflex eye movement, and reduced VOR adaptation occur in the RP-deficient mice (Tanaka et al., 2013). Considering similarities in induction conditions (Kawaguchi and Hirano, 2013) and suppressive effects on Purkinje cell activity between RP and LTD, they might synergistically support motor learning.

CONCLUSION

Postsynaptic depolarization of a cerebellar Purkinje cell induces long-term potentiation (LTP) of GABAergic inhibitory synaptic transmission which is called RP. Induction of RP depends on Ca^{2+}, CaMKII, GABARAP etc., and intricate regulatory mechanisms have been delineated. Transgenic mice defective in RP show defects in adaptation of VOR, indicating involvement of RP in motor learning.

ACKNOWLEDGMENTS

We thank Drs. Y. Tagawa, H. Tanaka and E. Nakajima for their constructive comments on the manuscript and Ms. Y. Tanaka for preparation of figures.

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

Received: 22 January 2014; paper pending published: 09 January 2014; accepted: 29 January 2014; published online: 18 February 2014.
Citation: Hirano T and Kawaguchi SY (2014) Regulation and functional roles of rebound potentiation at cerebellar stellate cell—Purkinje cell synapses. Front. Cell. Neurosci. ***8****:42. doi: 10.3389/fncel.2014.00042*
This article was submitted to the journal Frontiers in Cellular Neuroscience.

frontiers in
CELLULAR NEUROSCIENCE

ORIGINAL RESEARCH ARTICLE
published: 03 February 2014
doi: 10.3389/fncel.2014.00016

Distinct roles of α- and βCaMKII in controlling long-term potentiation of $GABA_A$-receptor mediated transmission in murine Purkinje cells

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Calcium/Calmodulin-dependent kinase type II (CaMKII) is essential for various forms of synaptic plasticity. The predominant α- and βCaMKII isoforms have both been shown to contribute to specific forms of plasticity at excitatory synapses, but little is known about their functions at inhibitory synapses. Here we investigated the role of both isoforms in long-term potentiation of the inhibitory molecular layer interneuron to Purkinje cell synapse (MLI-PC iLTP) upon climbing fiber (CF) stimulation. We demonstrate that deleting either the α- or βCaMKII isoform affected MLI-PC iLTP. In the presence of the PP2B blocker cyclosporin A, CF stimulation elicited iLTP in *Camk2b*$^{-/-}$ mice, but not in *Camk2a*$^{-/-}$ mice. Moreover, co-activation of the MLIs and CF suppressed iLTP in wild-type mice through activation of $GABA_B$-receptors, whereas it evoked iLTP in *Camk2b*$^{-/-}$. This reversal of the effect of αCaMKII activity in *Camk2b*$^{-/-}$ mutants upon co-activation did not critically involve protein kinase A, but depended on calcium release from internal stores. Our results indicate that α- and βCaMKII isoforms in Purkinje cells can be differentially activated and serve distinct roles in controlling iLTP. We propose that the CaMKII holo-enzyme may be selectively activated by various $GABA_B$-mediated pathways and that the presence of the βCaMKII isoform determines their impact on inhibitory plasticity.

Keywords: CaMKII, plasticity, GABAAR

INTRODUCTION

Calcium/Calmodulin-dependent Kinase type II (CaMKII) is one of the most densely expressed proteins in the central nervous system (Erondu and Kennedy, 1985). The intracellular signaling pathways that are controlled by CaMKII have been shown to be important for memory formation by controlling synaptic plasticity (Silva et al., 1992a,b; Colbran and Brown, 2004; Wayman et al., 2008). The CaMKII holo-enzyme is essential for pre- and post-synaptic mechanisms at both excitatory and inhibitory synapses in hippocampal, amygdalar, cortical, and cerebellar neurons (Castillo et al., 2011), which highlights the importance of this molecule for proper neuronal functioning.

In the brain the CaMKII holo-enzyme comprises predominantly α- and βCaMKII subunits (Miller and Kennedy, 1985). βCaMKII differs from αCaMKII by its actin binding domain and higher calcium sensitivity (Shen et al., 1998; Brocke et al., 1999; Thiagarajan et al., 2002; Fink et al., 2003; Cho et al., 2007). Recent studies revealed that each isoform has a distinct function in controlling synaptic plasticity at excitatory synapses in the neurons that express both α- and βCaMKII. For instance, deletion of αCaMKII results in disrupted long-term depression (LTD) at the excitatory granule cell – Purkinje cell synapse, whereas the deletion of βCaMKII bidirectionally reverses LTD and long-term potentiation (LTP; Hansel et al., 2006; van Woerden et al., 2009).

The molecular mechanisms that underlie long-term plasticity at inhibitory and excitatory synapses show extensive overlap, but it remains to be elucidated whether α- and βCaMKII serve distinct functions in controlling plasticity at inhibitory synapses. The functional relevance of this form of plasticity for cerebellar learning has been previously predicted (Wulff et al., 2009; Gao et al., 2012). Indeed, Tanaka et al. (2013) recently showed that it is involved in adaptation of the vestibulo-ocular reflex, which is controlled by the flocculus of the cerebellum. Here we studied the impact of genetic ablation of αCaMKII or βCaMKII on the expression of synaptic plasticity at the inhibitory molecular layer interneuron – Purkinje cell (MLI-PC) synapses using *Camk2a*$^{-/-}$ and *Camk2b*$^{-/-}$ mutant mice. Our results show that α- and βCaMKII isoforms serve distinct roles in controlling LTP at this inhibitory synapse (iLTP).

Abbreviations: AC, adenylyl cyclase; CaMKII, Calcium/Calmodulin-dependent kinase type II; CF, Climbing fiber; D-AP5, D(−)-2-amino-5-phosphonopentanoic acid; GABA, γ-aminobutyric acid; iLTP, inhibitory long-term potentiation; IP3, inositol-tri-phosphate; IPSC, inhibitory postsynaptic current; IPSP, inhibitory post-synaptic potential; LTP, long-term potentiation; MLI-PC, molecular layer interneuron – Purkinje cell; NBQX, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzoquinoxaline; PKA, protein kinase A; PP1, Phosphoprotein phosphatase 1; PP2B, protein-phosphatase type IIB; PTX, picrotoxin; TPRC, transient receptor potential canonical.

MATERIAL AND METHODS

ETHICAL APPROVAL

All studies were performed in accordance with the guidelines for animal experiments of the Erasmus Medical Center and the Dutch national legislation. All experiments and analyses were performed by scientists blinded to the genotype of the mouse.

ANIMALS

Camk2a$^{-/-}$ mice were generated as previously described (Elgersma et al., 2002) and for *Camk2b*$^{-/-}$ we used exon 2 knock-out mice, which showed complete loss of βCaMKII expression and ataxia, as described previously for the *Camk2b* exon 11 knock-out (van Woerden et al., 2009). Homozygous mice and wt littermates (both genders; generated by heterozygous × heterozygous breeding) ranging from postnatal day (P) 17–21 were used in all experiments. Animals were maintained at 22 ± 2°C with 12 h dark and light cycle and were provided with food and water *ad libitum*.

SLICE PREPARATION FOR ELECTROPHYSIOLOGY

Camk2a$^{-/-}$ and *Camk2b*$^{-/-}$ mice and wt littermates were decapitated under isoflurane anesthesia. Subsequently, the cerebellum was removed and transferred into ice-cold slicing medium that contains (in mM): 240 Sucrose, 5 KCl, 1.25 Na_2HPO_4, 2 $MgSO_4$, 1 $CaCl_2$, 26 $NaHCO_3$, and 10 D-Glucose, bubbled with 95% O_2 and 5% CO_2. Parasagittal slices (250 μm thick) of the cerebellar vermis were cut using a vibratome (VT1000S, Leica) and kept in ACSF containing (in mM): 124 NaCl, 5 KCl, 1.25 Na_2HPO_4, 2 $MgSO_4$, 2 $CaCl_2$, 26 $NaHCO_3$, and 20 D-Glucose, bubbled with 95% O_2 and 5% CO_2 for >1 h at 34 ± 1°C before the experiments started.

WHOLE-CELL ELECTROPHYSIOLOGY

Experiments were performed with a constant flow of oxygenated ACSF (1.5–2.0 ml/min). Purkinje cells were visualized using an upright microscope (Axioskop 2 FS plus, Carl Zeiss, Germany) equipped with a 40X water immersion objective. Patch-clamp recordings were performed using an EPC-10 double amplifier (HEKA electronics, Lambrecht, Germany). All recordings were performed at 34 ± 1°C.

Whole cell current clamp recordings of Purkinje cells were performed using borosilicate pipettes (R_{pip} = 2–4 MΩ) filled with intracellular solution containing (in mM): 130 K-Gluconate, 10 KOH, 3.48 $MgCl_2$, 4 NaCl, 10 HEPES, 4 Na_2ATP, 0.4 Na_3GTP, and 17.5 sucrose (pH 7.25, osmolarity 295). GABAergic MLI-PC synapses were stimulated as previously described (Mittmann and Häusser, 2007). In short, one patching pipette filled with ACSF was located at the molecular layer >200 μm lateral from Purkinje cells to avoid activating parallel fiber-Purkinje cell synapses. Our conditions resulted in a reversal potential for IPSPs of −75 to −78 mV with corrected liquid junction potentials. IPSPs were completely blocked by bath-applied non-competitive $GABA_A$-receptor blockers picrotoxin (100 μM) or SR95531 (10 μM). Evoked IPSPs from MLI-PC synapses appeared to be all or none, suggesting direct stimulations at stellate cell somata. To avoid intrinsically generated action potentials, Purkinje cells were kept at −60 to −65 mV with hyperpolarizing current injections (<−250 pA). Under these conditions, MLI-PC IPSPs appeared as negative potentials ranging from −0.2 to −3 mV. Climbing fibers (CFs) were stimulated with a patch electrode filled with external solution located in the granule cell layer. To induce LTP of MLI-PC IPSPs (i.e., iLTP), a tetanus of five CF stimuli at 10 Hz was applied every 2 s for 3 min. For paired MLI-CF stimulation, each CF stimulus was coincided with two MLI stimuli, i.e., at 20 Hz. Purkinje cell holding current and input resistance were constantly monitored, and cells with >15% shift of these parameters during the recording were excluded from analysis.

PURKINJE CELL SPONTANEOUS IPSCs AND REBOUND POTENTIATION

In a subset of recordings Purkinje cells were voltage clamped at −60 mV using intracellular solution containing (in mM): 150 CsCl, 15 CsOH, 1.5 $MgCl_2$, 0.5 EGTA, 10 HEPES, 4 Na_2ATP, and 0.4 Na_3GTP (pH 7.3; osmolarity 300). Ten μM NBQX was supplemented in the ACSF to avoid contamination with spontaneous EPSCs. Spontaneous IPSCs were analyzed using Minianalysis (Synaptosoft, Decatur, USA). To analyze IPSC kinetics, unitary IPSCs of 50–100 pA were selected to avoid interference of noise or insufficient voltage clamp. Traces were scaled, averaged and fit using a single decay time constant. Series and input resistances were monitored every 3 min using hyperpolarizing voltage steps; recordings were terminated if the holding current or the series or input resistances changed >15%.

PHARMACOLOGY

Baclofen (2 μM), cyclosporin A (5 μM), KN-93 (2 μM), SCH50911 (10 μM), KT 5720 (0.2 μM), and thapsigargin (10 μM) were obtained from Tocris Biosciences (Bristol, UK). Other chemicals were obtained from Sigma unless stated otherwise.

STATISTICS

To test for statistically significant differences between wt and *Camk2a*$^{-/-}$ and *Camk2b*$^{-/-}$ recordings we used an unpaired, two-way Student's *t*-test or a non-parametric Mann-Whitney *U* test depending on the distribution of the data. The level of significance ($p < 0.05$ or <0.001) is reported in the figure legends. To test whether a stimulus pattern induced a significant change we used a paired, two-way Student's *t*-test on the last 5 min before the tetanus (pre-tetatnus) and the 20–25 min after the tetanus (post-tetanus). For these latter comparisons we considered *p*-values <0.05 to be significant.

RESULTS

BOTH α- AND βCaMKII SUBUNITS ARE ESSENTIAL FOR iLTP AT THE MLI-PC SYNAPSE

To elucidate how α- and βCaMKII subunits mediate inhibitory synaptic plasticity, we investigated iLTP at MLI-PC synapses in *Camk2a*$^{-/-}$ and *Camk2b*$^{-/-}$ mice. To induce iLTP at MLI-PC synapses we activated the CF 5 times at 10 Hz every 2 s for 3 min (**Figure 1A**, inset); this tetanus significantly increased the MLI-IPSP amplitude in wild type (wt) Purkinje cells (averaged IPSP amplitude 20–25 min after the CF stimulus protocol (post-tetanus) was 138.2 ± 7.5% relative to the last 5 min pre-tetanus; $p = 0.0002$, **Figure 1A**). This iLTP occurred without inducing

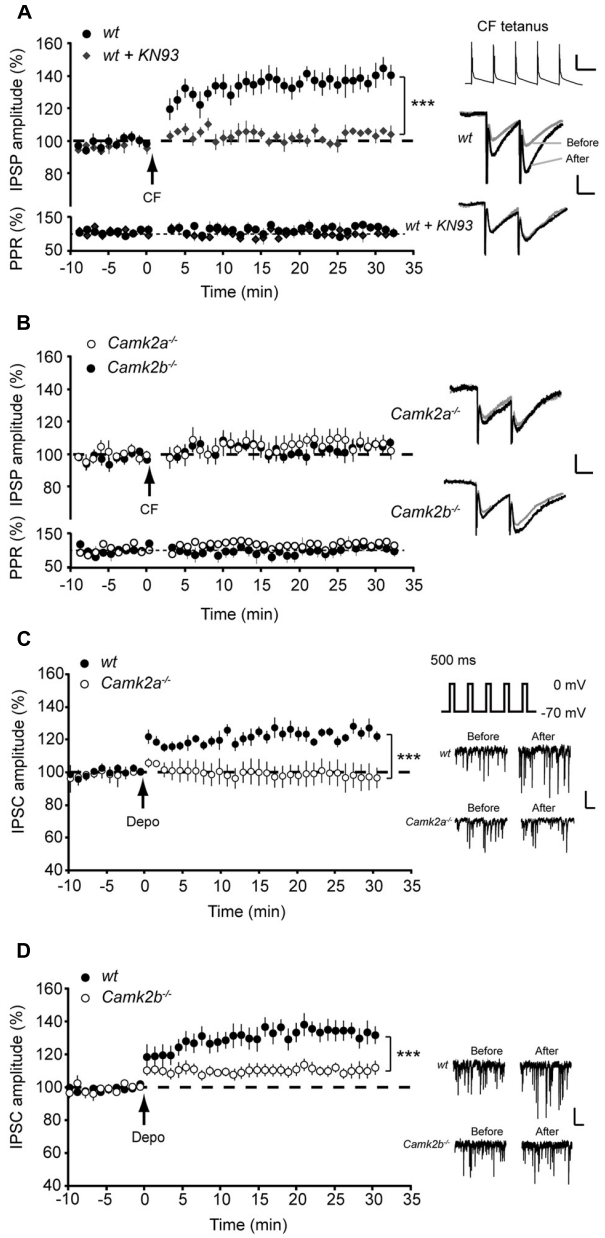

FIGURE 1 | Aberrant iLTP at MLI-Purkinje cell synapses in α and βCaMKII knockout mice. (**A**; Top) Five pulses, 10 Hz climbing fiber (CF) stimulation repeated every 2 s for 3 min yields iLTP in wildtype (wt, $n = 10$) Purkinje cells but not in wt Purkinje cells in the presence of KN-93 (wt + KN93, $n = 6$). (Bottom) Accompanying paired pulse ratio of IPSPs. Inset: representative traces of CF stimulation, scale bars 20 mV/100 ms and representative traces of IPSP before and after the CF stimulation, scale bars 1 mV/25 ms. **(B)** CF stimulation did not induce iLTP in *Camk2a*$^{-/-}$ ($n = 9$) and *Camk2b*$^{-/-}$ Purkinje cells ($n = 8$). **(C)** Inset shows schematic drawing of rebound potentiation experiment and representative traces of sIPSC, scale bars 50 pA/200 ms. Rebound potentiation in Purkinje cells was induced by five 500 ms depolarization pulses to 0 mV at 0.5 Hz; IPSC amplitudes were compared before and after tetanus. Impaired rebound potentiation in *Camk2a*$^{-/-}$ mice (wt, $n = 7$; *Camk2a*$^{-/-}$, $n = 6$). **(D)** Impaired rebound potentiation in *Camk2b*$^{-/-}$ mice (wt, $n = 8$; *Camk2b*$^{-/-}$, $n = 9$). Error bars represent SEM. Asterisks with brackets indicate statistical significance between wt and knockout mice (Student's *t*-test of averages over last 5 min). ***$p < 0.005$.

significant changes in the paired pulse ratio of two consecutive IPSPs with 50 ms interval ($p = 0.18$; **Figure 1A**), strongly suggesting that the site of plasticity was most likely postsynaptic. In accordance to previous reports that showed how the potentiation of inhibitory synaptic currents was fully blocked in wt by bath application of the global CaMKII blocker KN-93 (Kano et al., 1992; Kawaguchi and Hirano, 2000), our iLTP-induction protocol failed to induce a significant change in the postsynaptic responses to MLI stimulation (103.5 ± 2.1% relative to pre-tetanus; $p = 0.18$; **Figure 1A**). When the same CF stimulus protocol was delivered to either *Camk2a*$^{-/-}$ or *Camk2b*$^{-/-}$ Purkinje cells, we observed a significantly lower level of potentiation (108.8 ± 3.4 and 106.4 ± 2.5% of baseline IPSP amplitude, respectively; **Figure 1B**) than in wt Purkinje cells ($p = 0.0005$ and $p = 0.0006$; **Figures 1A,B**). Several possibilities could account for the reduction of iLTP induction in Purkinje cells of *Camk2a*$^{-/-}$ or *Camk2b*$^{-/-}$ mice. First, it is possible that deleting α- or βCaMKII induces a change in the surface level of $GABA_A$-receptors and thus precludes the induction of iLTP in response to CF stimulation. This is unlikely, however, since the frequency, amplitude, and kinetics of spontaneously occurring (s)IPSCs in Purkinje cells were not significantly different between *Camk2a*$^{-/-}$ and *Camk2b*$^{-/-}$ mice and their wt littermates (**Table 1A**). It is also unlikely that the lack of iLTP originates from aberrant CF stimulation since none of the response parameters evoked by such stimulus, i.e., the Na^+-spike, Ca^{2+}-spike, and Ca^{2+}-plateau amplitudes, was significantly different in *Camk2a*$^{-/-}$ and *Camk2b*$^{-/-}$ compared to their wt littermates (**Table 1B**). To test whether the lack of α- and βCaMKII prevents sufficient CF-stimulus induced Ca^{2+}-influx to activate the molecular machinery underlying iLTP, we tested whether another trigger of Ca^{2+}-influx could induce plasticity of spontaneously occurring IPSCs. Direct depolarization by voltage-clamping the Purkinje cell to 0 mV from a holding potential of −70 mV has been shown previously to effectively induce potentiation of sIPSCs in Purkinje cells (Kano et al., 1992). Five 500 ms depolarizing pulses from −70 to 0 mV with a 2 s interval readily potentiated sIPSCs in wt Purkinje cells, but not in *Camk2a*$^{-/-}$ Purkinje cells ($p = 0.002$, **Figure 1C**). Similarly, the iLTP induced by depolarization was significantly reduced in *Camk2b*$^{-/-}$ compared to wt ($p = 0.001$, **Figure 1D**). Together the effects of CF stimulation on MLI-IPSPs and of Purkinje cell depolarization on sIPSCs imply that both α- and βCaMKII are essential for post-synaptic iLTP at inhibitory synapses of Purkinje cells.

BLOCKING PP2B ACTIVITY RESCUES iLTP IN *Camk2b*$^{-/-}$ BUT NOT IN *Camk2a*$^{-/-}$ MICE

Following direct post-synaptic depolarization or CF activity the calcium concentration rises in Purkinje cells, which activates not only α- and βCaMKII but also protein phosphatase 2B (PP2B), the latter of which counteracts the effects of CaMKII activation (Kawaguchi and Hirano, 2002; Belmeguenai and Hansel, 2005; van Woerden et al., 2009). In order to test whether the residual CaMKII in *Camk2a*$^{-/-}$ and *Camk2b*$^{-/-}$ mutants is outcompeted by PP2B we examined the effect of the specific PP2B blocker cyclosporin A on iLTP evoked by CF stimulation. Inhibiting PP2B activity did not alter the level of iLTP in wt and *Camk2a*$^{-/-}$ Purkinje cells

Table 1 | Normal spontaneous IPSC properties in *Camk2a*$^{-/-}$ and *Camk2b*$^{-/-}$ Purkinje cells.

(A) sIPSC properties in *Camk2a*$^{-/-}$ and *Camk2b*$^{-/-}$ mice						
	#	**FF (Hz)**	**Amp (pA)**	**Rise (ms)**	**Decay (ms)**	**Width (ms)**
wt	12	18.4 ± 1.7	62.8 ± 4.0	1.28 ± 0.1	15.1 ± 1.1	2.8 ± 0.2
Camk2a$^{-/-}$	10	18.5 ± 0.8	65.1 ± 7.6	1.31 ± 0.1	13.7 ± 1.0	3.0 ± 0.2
t-tests		0.97	0.79	0.79	0.35	0.38
wt	13	19.2 ± 2.8	66.3 ± 3.0	1.0 ± 0.1	13.7 ± 0.9	2.6 ± 0.2
Camk2b$^{-/-}$	12	19.0 ± 2.8	64.4 ± 2.5	1.0 ± 0.1	13.2 ± 0.6	2.6 ± 0.1
t-tests		0.97	0.64	0.63	0.61	0.97
(B) Complex spike properties in *Camk2a*$^{-/-}$ and *Camk2b*$^{-/-}$ mice						
	#	**Na^+ spike (mV)**	**1st Ca^{2+} spike (mV)**	**Ca^{2+} plateau (mV)**	**AHP (mV)**	
wt	18	86.8 ± 4.8	56.8 ± 3.6	18.8 ± 1.0	−2.6 ± 0.2	
Camk2a$^{-/-}$	14	84.7 ± 2.9	51.4 ± 2.7	20.1 ± 1.7	−2.6 ± 0.2	
t-tests		0.72	0.25	0.49	0.70	
wt	16	85.8 ± 2.0	51.4 ± 1.8	21.6 ± 1.7	−2.6 ± 0.2	
Camk2b$^{-/-}$	15	81.5 ± 2.9	55.5 ± 1.6	21.2 ± 0.7	−2.3 ± 0.3	
t-tests		0.22	0.10	0.65	0.43	

(A) *Normal spontaneous IPSC properties in Camk2a*$^{-/-}$ *and Camk2b*$^{-/-}$ *Purkinje cells. Table: quantification and comparison of spontaneous IPSC frequency (FF), amplitude (Amp), and kinetics (10–90% rise time, decay time constant and width at 50% of the maximal amplitude) of IPSCs.* ***(B)*** *Normal complex spike properties in Camk2a*$^{-/-}$ *and Camk2b*$^{-/-}$ *Purkinje cells. Table: quantification and comparison of the amplitudes of Na^+ spike, the first Ca^{2+} spike (1st Ca^{2+} spike), the Ca^{2+} plateau, and after hyperpolarization (AHP) of the CF-EPSC in wt, Camk2a*$^{-/-}$ *and Camk2b*$^{-/-}$ *Purkinje cells.*

(129.9 ± 5.2 and 95.8 ± 4.9% compared to baseline IPSP amplitude, respectively; **Figure 2A**; $p = 0.14$ and 0.72 when compared to the condition without cyclosporin A as represented in **Figure 1A**). However, in *Camk2b*$^{-/-}$ the presence of cyclosporin A the CF stimulus protocol resulted in a significant iLTP comparable to that recorded in the wt cells (129.3 ± 5.9 and 128.8 ± 5.2%, respectively; $p = 0.95$; **Figure 2B**). These data suggest that the residual αCaMKII in the *Camk2b*$^{-/-}$, but not the residual βCaMKII in the *Camk2a*$^{-/-}$ mice, enables iLTP induction when PP2B is blocked.

CO-ACTIVATION OF MLIs AND CF FACILITATES iLTP IN *Camk2b*$^{-/-}$ BUT NOT IN *Camk2a*$^{-/-}$ MICE

Our results show that when PP2B activity is chemically blocked α- and βCaMKII serve differential roles during iLTP in Purkinje cells. One physiologically relevant cascade that mediates the impact of PP2B on iLTP at the MLI-PC synapse is controlled by activity of MLI's during CF-stimulation (Kawaguchi and Hirano, 2000). Here we paired the five pulses of 10 Hz CF stimulation with 10 pulses of 20 Hz MLI stimulation (see inset **Figure 3A**). This paired stimulation suppressed iLTP at the MLI-PC synapses in both wt groups to levels not significantly different from *Camk2a*$^{-/-}$ and *Camk2b*$^{-/-}$ (103.6 ± 5.2%, $p = 0.22$ and 102.5 ± 3.7%, $p = 0.52$, respectively; **Figures 3A,B**), without changing the paired pulse ratio (paired Student's *t*-test of averages pre- vs. post-tetanus: all *p*-values > 0.7). In *Camk2a*$^{-/-}$ this suppression protocol did not induce a significant change in synaptic strength (97.8 ± 4.1% of the pre-tetanus IPSP amplitude; $p = 0.29$; **Figure 3A**), whereas in *Camk2b*$^{-/-}$ the same conditions evoked iLTP (132.2 ± 4.8%; $p = 0.0002$; **Figure 3B**). We next tested whether this unexpected expression of iLTP in *Camk2b*$^{-/-}$ mice in response to a stimulation protocol that suppresses iLTP in wt mice was dependent on the activity of residual αCaMKII. We repeated the paired MLI-CF protocol in the presence of KN-93 in *Camk2b*$^{-/-}$ mice. Indeed, in this condition, i.e., when all residual CaMKII activity is blocked, no detectable iLTP was found in *Camk2b*$^{-/-}$ mice (103.0 ± 5.4% of the pre-tetanus IPSP amplitude; $p = 0.60$; **Figure 3B**). These results indicate that in the absence of βCaMKII MLI-CF stimulation induced iLTP by activation of residual αCaMKII.

$GABA_B$-RECEPTOR ACTIVATION FACILITATES iLTP IN *Camk2b*$^{-/-}$ BUT NOT IN *Camk2a*$^{-/-}$ MICE

The molecular mechanism underlying the effect of MLI stimulation on Ca^{2+}- and CaMKII-dependent potentiation of inhibitory responses in Purkinje cells have been linked to $GABA_B$-receptor activation (Kawaguchi and Hirano, 2000; Kawaguchi and Hirano, 2002). To study whether the $GABA_B$-receptor activation is essential for iLTP, we next blocked the $GABA_B$-receptor activation with SCH 50911 during the paired MLI-CF stimulation protocol. Under these conditions the MLI-CF stimulation did not evoke iLTP in *Camk2b*$^{-/-}$ (101.7 ± 8.9% of the pre-tetanus IPSP amplitude; $p = 0.69$; **Figure 3C**). The efficacy of this approach is indicated by the fact that in wt SCH 50911 cancelled the suppression effect of co-activating the MLI-CF inputs, i.e., iLTP could be induced (133.3 ± 8.4% of the pre-tetanus IPSP amplitude; $p = 0.002$; **Figure 3C**). To study whether $GABA_B$-receptor activation paired with CF stimulation is also

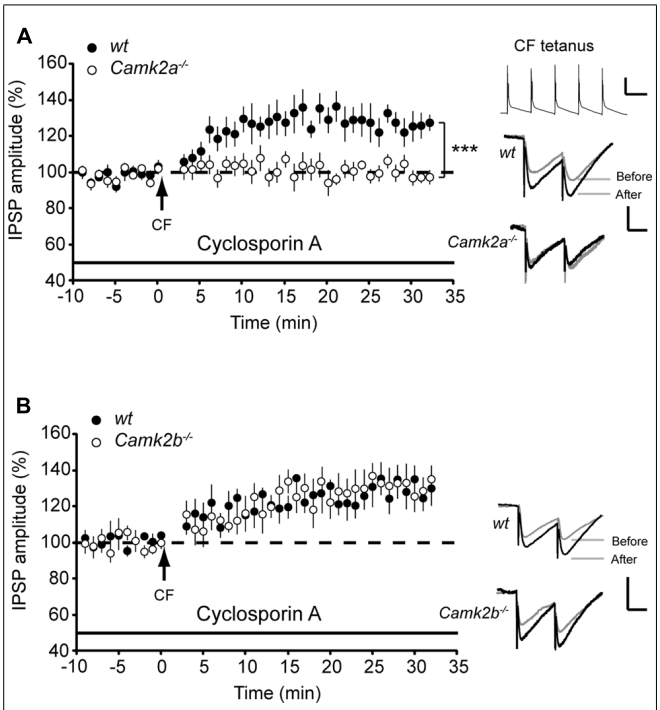

FIGURE 2 | Block of PP2B reveals iLTP and block of IP_3 disrupts rescue of iLTP in *Camk2b*$^{-/-}$ mice. (A) CF stimulation yields iLTP in wt Purkinje cells ($n = 7$) in the presence of 5 μM PP2B inhibitor cyclosporin A, but not in *Camk2a*$^{-/-}$ Purkinje cells ($n = 10$). Inset: representative traces of CF stimulation, scale bars 20 mV/100 ms and representative traces of IPSP before and after the CF stimulation, scale bars 1 mV/25 ms). **(B)** CF stimulation yields iLTP in wt Purkinje cells ($n = 6$) in the presence of 5 μM PP2B inhibitor cyclosporin A, as well as in *Camk2b*$^{-/-}$ Purkinje cells ($n = 8$). Inset: representative traces of CF stimulation, scale bars 20 mV/100 ms and representative traces of IPSP before and after the CF stimulation, scale bars 1 mV/25 ms. Error bars represent SEM. Asterisks with brackets indicate statistical significance between wt and knockout mice (Student's *t*-test of averages over last 5 min). ***$p < 0.005$.

sufficient to evoke iLTP in *Camk2b*$^{-/-}$ mutants we replaced the MLI stimulation with the bath-applied $GABA_B$-receptor agonist Baclofen. In the presence of Baclofen CF stimulation evoked iLTP in *Camk2b*$^{-/-}$ (121.0 ± 4.1% of the pre-tetanus IPSP amplitude; $p = 0.002$) and suppressed iLTP in wt (101.5 ± 2.7%; $p = 0.61$; **Figure 3D**). Together these experiments unequivocally show that in wt $GABA_B$-receptor activation suppresses CF-evoked iLTP, but that in *Camk2b*$^{-/-}$ $GABA_B$-receptor activation is both essential but also sufficient to facilitate iLTP evoked by CF-activity.

CaMKII SUBUNITS MAY DIFFERENTIATE THE EFFECTS OF $GABA_B$-RECEPTORS ON iLTP

How can $GABA_B$-receptor activation inhibit iLTP in wt and facilitate iLTP in *Camk2b*$^{-/-}$ mice? It is known that $GABA_B$-receptor activation mediates the activity of two separate pathways (**Figure 4A**); upon $GABA_B$-receptor activation protein kinase A (PKA) is inhibited, which promotes the PP2B-dependent suppression of the CaMKII-mediated iLTP (Kawaguchi and Hirano, 2002); and $GABA_B$-receptor activation induces calcium release from internal stores, which could promote iLTP (Komatsu, 1996;

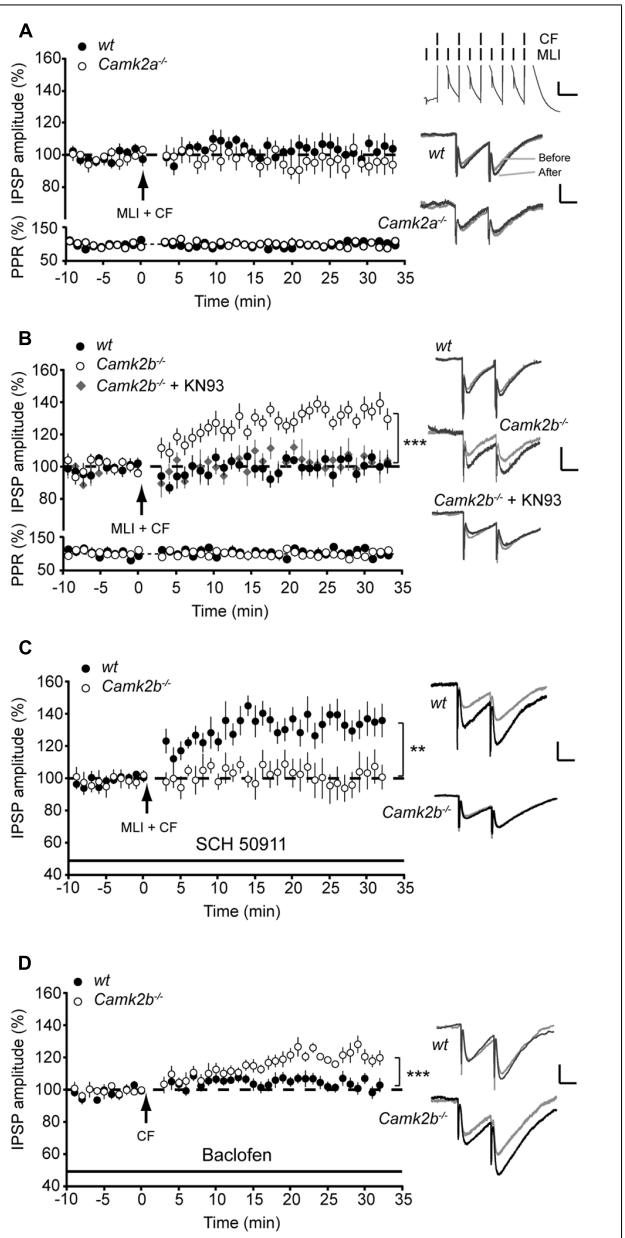

FIGURE 3 | $GABA_B$-receptor activation facilitates iLTP in *Camk2b*$^{-/-}$ but not in *Camk2a*$^{-/-}$ mice. (**A**; Top) Paired molecular layer interneuron (MLI; 20 Hz) and climbing fiber (CF; 5 pulses, 10 Hz) stimulation every 2 s for 3 min yields no change in IPSP amplitude in both wt ($n = 10$) and *Camk2a*$^{-/-}$ ($n = 8$) Purkinje cells. (Bottom) Accompanying paired pulse ratio of IPSPs. Inset: representative traces of MLI-CF stimulation, scale bars 3 mV/100 ms and representative traces of IPSP before and after the CF stimulation, scale bars 1 mV/25 ms.**(B)** Paired MLI-CF stimulation yields no change in IPSP amplitude in wt Purkinje cells ($n = 10$) and in *Camk2b*$^{-/-}$ Purkinje cells when KN93 is present (*Camk2b*$^{-/-}$ + KN93, $n = 5$). MLI-CF stimulation significantly facilitates iLTP in *Camk2b*$^{-/-}$ Purkinje cells ($n = 13$). **(C)** Inhibition of $GABA_B$-receptor activation with SCH 50911 rescues iLTP in wt Purkinje cells ($n = 9$), but inhibits iLTP in *Camk2b*$^{-/-}$ Purkinje cells ($n = 7$) following paired SC–CF stimulation. **(D)** Activation of $GABA_B$-receptors with baclofen inhibits iLTP in wt Purkinje cells ($n = 10$), but facilitates iLTP in *Camk2b*$^{-/-}$ cells ($n = 8$) following CF stimulation. Error bars represent SEM. Asterisks with brackets indicate statistical significance between wt and knockout mice (Student's *t*-test of averages over last 5 min). **$p < 0.01$; ***$p < 0.005$.

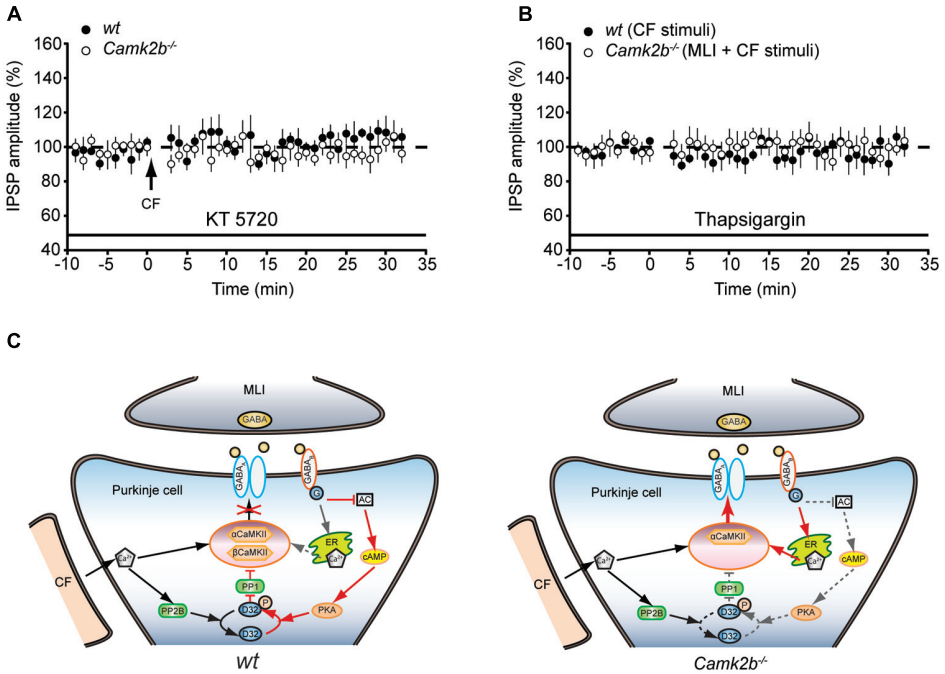

FIGURE 4 | $GABA_B$-receptor activationmay operate two distinct pathways to activate or inhibit the iLTP induction. (A) Left, schematic representation of the working model of CaMKII mediated iLTP induction cascade and $GABA_B$-receptor mediated inhibition of iLTP in wt Purkinje cell. The model is proposed based on previous studies (Kano et al., 1996; Kawaguchi and Hirano, 2002) and the current data. Cascades are simplified for the clarity of illustration. Arrows indicate activation cascades, bars indicate inhibitory cascades. Note that in the presence of both α and βCaMKII, the calcium release from internal stores upon $GABA_B$-receptor activation is outcompeted by the suppressing PKA-PP1 pathway (dashed arrow). AC, adenylyl cyclase; D32, DARPP-32. Right, schematic representation of the CaMKII mediated iLTP induction cascade and $GABA_B$-receptor mediated inhibition of iLTP in *Camk2b*$^{-/-}$ Purkinje cells. Genetic deletion of βCaMKII revealed a rescue of iLTP by $GABA_B$-receptor activation. Note that (1) the inhibitory effect of PKA-PP1 pathway upon $GABA_B$-receptor activation is minimized (indicated in dashed lines) in the absence βCaMKII and that (2) the facilitating effects of calcium release from internal stores enables the rescue of iLTP **(B)** Inhibition of PKA with KT5720 suppresses iLTP in wt Purkinje cells ($n = 5$), but does not rescue iLTP in *Camk2b*$^{-/-}$Purkinje cells ($n = 6$) following CF stimulation. **(C)** Inhibition of calcium release from internal stores with thapsigargin abolishes the facilitation of iLTP in *Camk2b*$^{-/-}$ Purkinje cells ($n = 7$) following paired MLI-CF stimulation, as well as iLTP in wt Purkinje cells ($n = 6$) following CF stimulation. Error bars represent SEM. Asterisks with brackets indicate statistical significance between wt and knockout mice.

Yamauchi, 2005). We hypothesized that the presence of βCaMKII determines which of these pathways prevails and thereby whether upon MLI-CF co-activation iLTP is induced or not. To test this working hypothesis, we first assessed whether $GABA_B$-mediated inhibition of PKA, which is critical for the suppression of iLTP in wt (Kawaguchi and Hirano, 2002), also mediates the rescue of iLTP in *Camk2b*$^{-/-}$. However, the presence of the PKA blocker KT5720 did not result in a rescue of iLTP in *Camk2b*$^{-/-}$ following CF stimulus (97.4 ± 7.0% of the pre-tetanus IPSP amplitude; $p = 0.61$) whereas it did block iLTP in wt Purkinje cells [106.22 ± 0.83%; $p = 0.68$; **Figure 4B**; see also (Kawaguchi and Hirano, 2002)]. These results indeed indicate that $GABA_B$-receptor activation facilitates iLTP in *Camk2b*$^{-/-}$ by a separate pathway that is PKA-independent. To test whether instead of the PKA-pathway the $GABA_B$-mediated calcium release from internal stores controls the rescue of iLTP in *Camk2b*$^{-/-}$ evoked by the suppression protocol, we tested the effect of thapsigargin, which depletes intracellular calcium stores. In the presence of thapsigargin, the suppression protocol failed to rescue iLTP in *Camk2b*$^{-/-}$ (94.6 ± 3.0% of the pre-tetanus IPSP amplitude; $p = 0.74$) and the CF protocol failed to induce iLTP in wt (99.9 ± 5.7%; $p = 0.88$; **Figure 4C**). Together these results support our working hypothesis that $GABA_B$-receptor activation suppresses iLTP in the presence of βCaMKII in a PKA-dependent manner, but rescues iLTP in the absence of βCaMKII by raising the intracellular calcium concentration through calcium release from internal stores.

DISCUSSION

The current study shows that αCaMKII and βCaMKII both play a role in induction of iLTP at MLI-PC synapses, but that both isoforms can be activated selectively and serve a distinct function in this process. Two lines of evidence support these conclusions. First, when the competing PP2B activity is blocked, iLTP is only expressed following CF stimulation in *Camk2b*$^{-/-}$, not in the *Camk2a*$^{-/-}$. Second, whereas co-activation of MLIs and CF suppresses iLTP in wt, this protocol permits iLTP induction in *Camk2b*$^{-/-}$. Thereby our results indicate that the presence of βCaMKII determines whether activation of αCaMKII evokes iLTP at MLI-PC synapses.

Several studies confirmed the involvement of the CaMKII holo-enzyme in synaptic plasticity at inhibitory synapses in the

hippocampus, amygdala, cerebral cortex, and cerebellum (Kano et al., 1996; Kawaguchi and Hirano, 2002; Bauer and LeDoux, 2004; Maffei et al., 2006; Xu et al., 2008; Houston et al., 2009; Castillo et al., 2011), but it has not yet been possible to decipher the individual contributions of α- and βCaMKII to iLTP. Given the overlap of molecular components between the signaling pathways that control synaptic plasticity at both excitatory and inhibitory synapses (Collingridge et al., 2004), one would predict distinct roles of α- and βCaMKII in iLTP much alike those recently described for excitatory synapses (Cho et al., 2007; van Woerden et al., 2009). Indeed, we found that in *Camk2b*$^{-/-}$ Purkinje cells the $GABA_B$-activation was essential to elicit iLTP upon CF stimulation, whereas in *Camk2a*$^{-/-}$ Purkinje cells $GABA_B$-activation did not trigger iLTP. Due to the impact of blocking calcium release on the expression of iLTP in *Camk2b*$^{-/-}$ these results seem in accordance with the predicted lower calcium sensitivity of αCaMKII in *Camk2b*$^{-/-}$ Purkinje cells than in wt *Camk2a*$^{-/-}$ Purkinje cells (Brocke et al., 1999). However, the fact that the same stimulus protocol can suppress iLTP in Purkinje cells when βCaMKII molecules are present argues against a dominant role of the enhanced calcium sensitivity of βCaMKII. An alternative possibility is that the actin-binding domain of βCaMKII may act as a differentiator: in *Camk2b*$^{-/-}$ mutants the residual αCaMKII is not confined to actin and thereby can be more readily activated by local calcium sources like intracellular calcium stores (Finch and Augustine, 1998), store-operated calcium influx, or activation of the transient receptor potential canonical (TPRC) channels, all of which promote CaMKII-mediated iLTP (Shen et al., 1998; Hirono et al., 2001; New et al., 2006; Xu et al., 2008; Chae et al., 2012). This hypothesis should be tested in future experiments, taking into account that any of these local calcium sources may be essential for iLTP induction as well (Komatsu, 1996).

Although our study focussed on the post-synaptic effects of the absence of either αCaMKII or βCaMKII, we cannot exclude the possibility that a presynaptic function of CaMKII, such as phosphorylating synapsin-1 and thereby enhancing neurotransmitter release, is also affected. Yet, our recordings on the spontaneous release and stimulus-evoked GABA release from MLI terminals and glutamate release from CF terminals in *Camk2a*$^{-/-}$ and *Camk2b*$^{-/-}$ does not show any significant difference (**Table 1**). Still, MLIs as well as neurons in the inferior olive express βCaMKII (but not αCaMKII; Hansel et al., 2006) and could therefore in principle be subject to affected neurotransmitter release in *Camk2b*$^{-/-}$. Since several studies have shown a role of CaMKII in neurotransmitter release from other cerebellar neurons such as granule cells (e.g., León et al., 2008), a more detailed study on the presynaptic effects of the lack of βCaMKII that focusses on the release probability in MLIs and CFs is warranted.

The induction rules for plasticity of inhibitory synapses at cerebellar MLI-PC synapses are opposite to those in early postnatal hippocampal CA1 tissue: coincident pre- and postsynaptic activity results in suppression of iLTP at MLI-PC synapses through $GABA_B$-receptor activation (Kawaguchi and Hirano, 2000), whereas this cascade is essential for iLTP at CA1 synapses (Xu et al., 2008). Our data show that genetic ablation of βCaMKII reverts the iLTP induction rules at cerebellar MLI-PC synapses to hippocampal CA1-like rules, in that coincident presynaptic activity is essential for the induction of iLTP in *Camk2b*$^{-/-}$. This surprising finding at this inhibitory synapse shows a remarkable coherence with the inversion of induction rules of long-term plasticity at excitatory parallel fiber – Purkinje cell synapses (van Woerden et al., 2009). Here too, the lack of βCaMKII reversed the induction rules for LTP and LTD, highlighting the overlap in molecular pathways of inhibitory and excitatory plasticity (Collingridge et al., 2004). Moreover, recent evidence indicates that local calcium concentrations control the selective translocation of αCaMKII molecules to either excitatory or inhibitory synapses in hippocampal tissue (Marsden et al., 2010), physically merging the molecular pathways that control plasticity at both types of synapses. Current studies promote a central role of βCaMKII in coordinating the translocation of CaMKII holoenzyme complexes in excitatory synapses in cerebellar Purkinje cells (van Woerden et al., 2009). Here, we have provided evidence that βCaMKII may also play a similar pivotal role at its inhibitory synapses.

AUTHOR CONTRIBUTIONS

Zhenyu Gao and Freek E. Hoebeek were involved in the conception and design of the experiments, data collection, analysis, and interpretation. All authors were involved in drafting and critical commenting on the manuscript. All authors approved this manuscript.

ACKNOWLEDGMENTS

We are grateful to M. Elgersma, E. Haasdijk, M. Rutteman, and P. Plak for technical assistance and E. Galliano, B. van Beugen, and N. Zuiderveen Borgesius for constructive discussions. This work was supported by grants from Erasmus University Rotterdam Fellowship program (Freek E. Hoebeek), NWO–ALW (Chris I. De Zeeuw, Ype Elgersma, Freek E. Hoebeek), NWO–ZON–MW (Chris I. De Zeeuw, Ype Elgersma, Freek E. Hoebeek), Neuro–BSIK (Chris I. De Zeeuw, Ype Elgersma), EEC–SENSOPAC-CEREBNET-C7 (Chris I. De Zeeuw), and ERC-advanced (Chris I. De Zeeuw).

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

Received: 14 November 2013; accepted: 10 January 2014; published online: 03 February 2014.

Citation: Gao Z, van Woerden GM, Elgersma Y, De Zeeuw CI and Hoebeek FE (2014) Distinct roles of α- and βCaMKII in controlling long-term potentiation of $GABA_A$-receptor mediated transmission in murine Purkinje cells. Front. Cell. Neurosci. ***8****:16. doi: 10.3389/fncel.2014.00016*

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

frontiers in
CELLULAR NEUROSCIENCE

HYPOTHESIS AND THEORY ARTICLE
published: 13 May 2014
doi: 10.3389/fncel.2014.00128

Plasticity of GABA transporters: an unconventional route to shape inhibitory synaptic transmission

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The brain relies on GABAergic neurons to control the ongoing activity of neuronal networks. GABAergic neurons control the firing pattern of excitatory cells, the temporal structure of membrane potential oscillations and the time window for integration of synaptic inputs. These actions require a fine control of the timing of GABA receptor activation which, in turn, depends on the precise timing of GABA release from pre-synaptic terminals and GABA clearance from the extracellular space. Extracellular GABA is not subject to enzymatic breakdown, and its clearance relies entirely on diffusion and uptake by specific transporters. In contrast to glutamate transporters, GABA transporters are abundantly expressed in neuronal pre-synaptic terminals. GABA transporters move laterally within the plasma membrane and are continuously trafficked to/from intracellular compartments. It is hypothesized that due to their proximity to GABA release sites, changes in the concentration and lateral mobility of GABA transporters may have a significant effect on the time course of the GABA concentration profile in and out of the synaptic cleft. To date, this hypothesis remains to be tested. Here we use 3D Monte Carlo reaction-diffusion simulations to analyze how changes in the density of expression and lateral mobility of GABA transporters in the cell membrane affect the extracellular GABA concentration profile and the activation of GABA receptors. Our results indicate that these manipulations mainly alter the GABA concentration profile away from the synaptic cleft. These findings provide novel insights into how the ability of GABA transporters to undergo plastic changes may alter the strength of GABAergic signals and the activity of neuronal networks in the brain.

Keywords: GABA, GABA transporters, GAT1, GAT3, uptake, diffusion, spillover, synaptic transmission

INTRODUCTION

There is a population of neurons in the mammalian brain that differs for their morphology, embryonic origin, connectivity and firing properties, but that shares the common ability to synthesize GABA, transport it into synaptic vesicles and release it in the synaptic cleft to communicate with post-synaptic target cells (Defelipe, 1993; Cauli et al., 1997; Gupta et al., 2000; Ascoli et al., 2008; Klausberger and Somogyi, 2008; Vitalis and Rossier, 2011). GABAergic neurons control the onset of large-scale network oscillations at various frequency ranges during development and in the mature brain, and their dysfunction is implicated with the onset of disease states like epilepsy, schizophrenia and autism (Le Magueresse and Monyer, 2013). In order to coordinate the activity of large neuronal ensembles, it is necessary to perfectly time GABA release from pre-synaptic terminals with GABA receptors activation in pre- and post-synaptic membranes, and GABA clearance from the extracellular space. There is no enzyme in the extracellular space that can convert GABA into a biologically inert molecule. As a consequence, GABA clearance relies entirely on diffusion and uptake by specific GABA transporters. As GABA diffuses away from its release site, it binds to synaptic and extra-synaptic receptors and to GABA transporters. Despite their name, GABA transporters do not always translocate across the cell membrane all the GABA molecules that they bind (i.e., they do not have 100% transport efficiency) but, in some cases, they can also release them back in the extracellular space (Bicho and Grewer, 2005). These events are reminiscent of those experienced by other neurotransmitters that are not subject to extracellular enzymatic degradation, like glutamate (Bergles et al., 1999).

One key difference, however, is that GABA and glutamate transporters have different cellular and sub-cellular distributions and different levels of expression (Zhou and Danbolt, 2013). With the exception of thalamic, Purkinje and striatonigral synapses, the highest level of expression of GABA transporters is found in neurons (Zhou and Danbolt, 2013). In contrast, glutamate transporters are abundantly expressed in astrocytes (Danbolt, 2001). In the hippocampus, the density of expression of GABA transporters is 800–1300 μm^{-2} (Chiu et al., 2002), considerably lower than that of glutamate transporters (10,800 μm^{-2} Lehre and Danbolt, 1998). This scarceness of GABA transporters could increase the likelihood of GABA spillover over that of glutamate in this brain region.

There are two main types of GABA transporters in the brain: GAT1 and GAT3. Neurons express GAT1, whereas astrocytes express GAT1 and GAT3 (Minelli et al., 1995,

1996). Immunocytochemistry experiments indicate that in neurons, GABA transporters are mainly localized in pre-synaptic GABAergic axon terminals (Radian et al., 1990; Ikegaki et al., 1994; Minelli et al., 1995; Conti et al., 1998; Zhou and Danbolt, 2013). One may consider this to be a strategic location, because it is the closest to GABA release sites. At excitatory synapses, glutamate transporters are located further away from the release sites, mainly in astrocytic processes adjacent to active synapses (Danbolt, 2001; Scimemi et al., 2009; Holmseth et al., 2012; Zhou and Danbolt, 2013). It is unclear whether the location of GABA transporters allows them to clear the released neurotransmitter more effectively than glutamate transporters. This could happen if the GABA transporters present inside the synaptic cleft were many and with rapid binding kinetics (see also Rusakov et al., 2011). It remains unclear whether GABA transporters can shape the GABA concentration profile inside the synaptic cleft, given what is currently known about their expression, binding kinetics, and transport efficiency.

Several experimental findings converge to indicate that GABA transporters in the cell membrane constitutively recycle to/from the cytoplasm (Deken et al., 2003; Wang and Quick, 2005) and move laterally within the lipid bilayer (Imoukhuede et al., 2009; Moss et al., 2009). Both phenomena are considered to be "rapid." The time constants of GABA transporters exo/endocytosis are 1.6 and 0.9 min, respectively (Wang and Quick, 2005) and the fluorescence recovery after photobleaching of surface expressed GAT1-YFP8 molecules occurs with a half time of ∼20 s (Imoukhuede et al., 2009). There are intracellular signaling cascades that can alter the number of GABA transporters expressed in the cell membrane and their recycling rate toward the cytosol (Whitworth and Quick, 2001a,b; Deken et al., 2003; Wang and Quick, 2005). Accordingly: PKC activation and tyrosine kinase inhibition cause a reduction in GABA uptake (Beckman et al., 1998, 1999; Law et al., 2000); depolarizing events that induce activation of voltage-gated Ca^{2+} channels increases the recycling rate of GABA transporters (Deken et al., 2003); proteins of the SNARE complex that mediate neurotransmitter vesicle release, like syntaxin 1A, interact with GABA transporters and increase their surface expression (Beckman et al., 1998; Deken et al., 2000). These findings provide evidence that the neuronal expression of GABA transporters can be modified within and across synapses depending on their level of activity. Likewise, the mobility of GABA transporters within the cell membrane can also be regulated by intracellular signaling cascades that involve PKC activation and that alter the interaction between GABA transporters and adapter proteins that anchor them to the cell cytoskeleton (Imoukhuede et al., 2009; Moss et al., 2009).

What is the effect of these modifications? How does the GABA concentration profile in the synaptic cleft and in the surrounding extracellular volume change, with different levels of expression and mobility of GABA transporters? Here we address this question by using 3D Monte Carlo reaction-diffusion simulations of GABA release from an active synapse. Our findings indicate that: (1) varying the concentration of GABA transporters alters activation of GABA receptors away from the release site, not of GABA receptors in the post-synaptic membrane directly opposed to it; (2) increasing the lateral mobility of GABA transporters facilitates GABA diffusion away from the synaptic cleft without altering the lifetime of GABA in the extracellular space. We analyze these effects during single and repeated stimulations. Taken together, these findings indicate that by altering the expression and diffusion of GABA transporters, the brain can control, in an activity-dependent manner, the spatial specificity of GABAergic signals.

MODEL DESCRIPTION: GEOMETRY AND SETTINGS

We used Blender 2.69 to design a simulation environment containing the 3D geometry of an average mouse hippocampal *stratum radiatum* axo-somatic GABAergic synapse (estimated by comparing the synaptic structure analysis from Nusser et al., 1997; Schikorski and Stevens, 1997; Biro et al., 2006; Specht et al., 2013). **Figure 1** provides an overview of the geometry of the simulation environment created *in silico* with Blender (**Figures 1A–C**), together with a schematic representation of the parameters that were tested (**Figures 1D–E**). The simulation environment consisted of a cube (11 μm wide), which we refer to as the "world." The world had a volume of $V_{world} = 1331\,\mu m^3$ and contained the soma of an ideal post-synaptic cell and the pre-synaptic terminal of an ideal GABAergic bouton. The portion of the world that was not occupied by the soma and the pre-synaptic terminal was referred to as the neuropil. The soma was represented as a sphere with the radius (r) of a typical hippocampal *stratum radiatum* interneuron ($r_{post} = 5\,\mu m$). The pre-synaptic terminal was represented as a hemisphere ($r_{pre} = 0.3\,\mu m$). The inner cleft area was modeled as a circle ($r_{icleft} = 0.1\,\mu m$) at the surface of the soma. The size of the inner cleft area matched the average size of the active zone region at small excitatory and inhibitory central synapses (Nusser et al., 1997; Schikorski and Stevens, 1997; Biro et al., 2006; Specht et al., 2013). The outer cleft area, which corresponds to the perisynaptic portion of the post-synaptic membrane, was represented as an annular region that extended for $r_{ocleft} = 0.2\,\mu m$ beyond the edge of the inner cleft area. We used CellBlender v1.0 (www.mcell.org) to simulate GABA release from the pre-synaptic terminal and diffusion in the extracellular space. At the beginning of each simulation, $n_{GABA} = 2000$ GABA molecules were released from the center of the flat region of the pre-synaptic terminal, in the inner volume of the synaptic cleft. When we monitored the effect of varying the density of expression of GABA transporters, we repeated each simulation for $n_{seed} = 100$ times; each simulation consisted of $n_{iter} = 50,000$ iterations with a time step of $\Delta t = 1\,\mu s$ (i.e., a total simulation time of 50 ms). Each simulation required a significantly longer computational time when we monitored the effect of varying the diffusion coefficient of GABA transporters. These simulations were repeated for $n_{seed} = 30$ times and each simulation consisted of $n_{iter} = 5000$ iterations with a time step of $\Delta t = 10\,\mu s$ (i.e., a total simulation time of 5 ms). We measured the free GABA concentration in the inner and outer cleft volume and in the neuropil. The GABA waveforms obtained in CellBlender were exported into ChanneLab2 (www.synaptosoft.com) to simulate the response of $GABA_A$ receptors. The majority of native $GABA_A$ receptors are thought to assemble as combinations of $\alpha\beta\gamma$ (here termed γ-subunit containing $GABA_A$

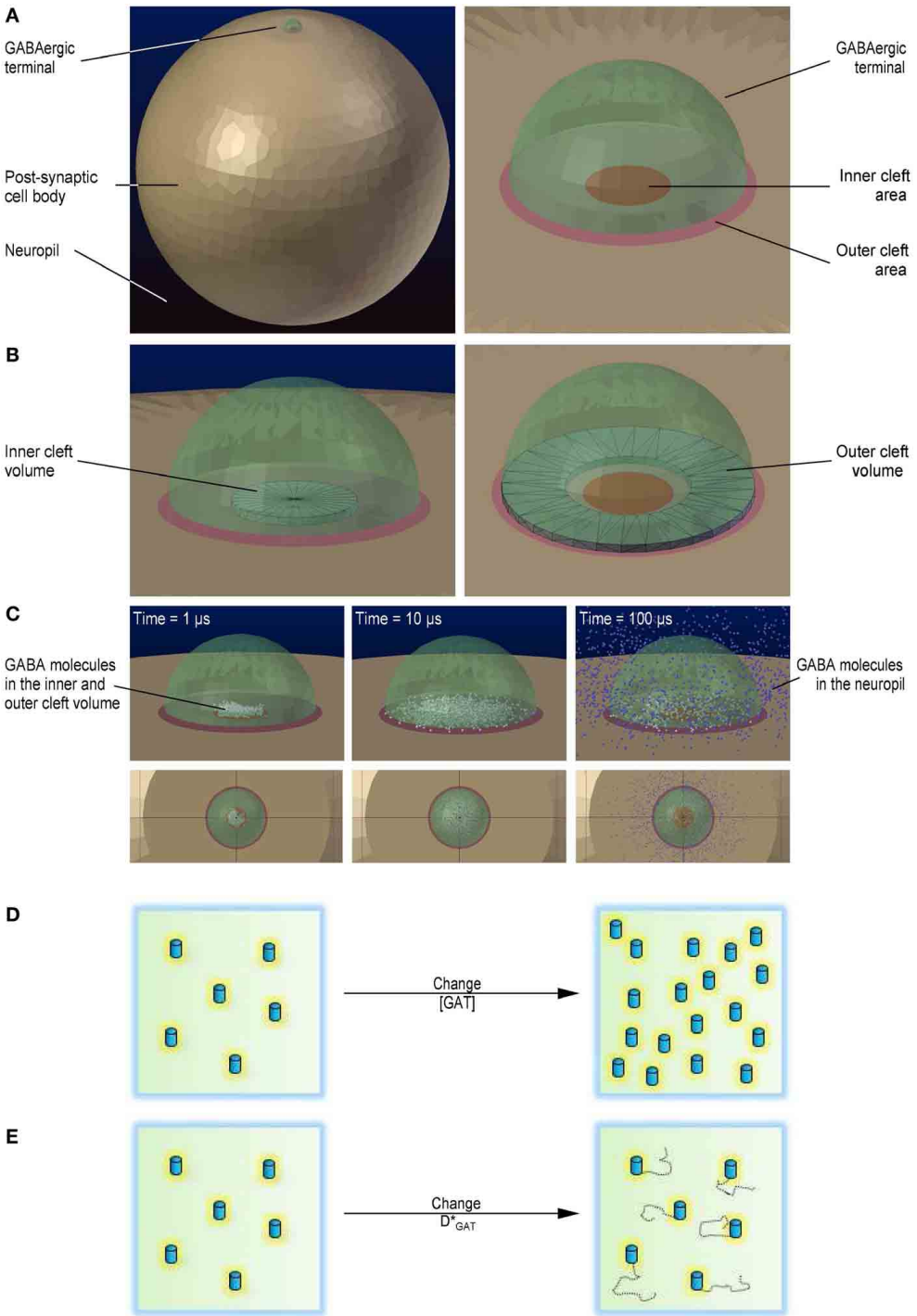

FIGURE 1 | 3D geometry of the modeled simulation environment. (A) Geometry of the simulation environment used to run the Monte Carlo reaction-diffusion simulations in CellBlender (left). The large light brown sphere represents the cell body of a post-synaptic cell. The small green, semi-transparent hemisphere represents an axo-somatic GABAergic synaptic terminal. The dark blue background represents the neuropil. Close-up view of the presynaptic terminal, including the inner and the outer cleft areas (right). The red circle represents the inner cleft area. The pale red annulus represents the outer cleft area. **(B)** The black wireframe shows the portion of the synaptic cleft volume above the inner cleft area in which we monitored the GABA concentration (left). The black wireframe shows the portion of the synaptic cleft volume above the perisynaptic region in which we monitored the GABA concentration (right). **(C)** Localization of GABA molecules diffusing away from their release site. GABA molecules diffusing within the inner and outer cleft volume are represented as white spheres. GABA molecules diffusing in the neuropil are represented as blue spheres. The three snapshots were obtained 1 µs (left), 10 µs (middle) and 100 µs after release (right). **(D)** Schematic diagram illustrating the change in GABA transporter concentration. **(E)** Schematic diagram illustrating the change in GABA transporter surface mobility.

receptors) or $\alpha\beta\delta$ subunits (here termed δ-subunit containing $GABA_A$ receptors) (Haas and Macdonald, 1999). There is evidence that δ-subunit containing $GABA_A$ receptors are mainly extra-synaptic, whereas γ-subunit containing $GABA_A$ receptors are present in synaptic and extra-synaptic regions (Nusser et al., 1998). Therefore, in our analysis, we compared the response of γ-subunit containing $GABA_A$ receptors in the inner cleft area and of γ- and δ-subunit containing $GABA_A$ receptors in the outer cleft area (Kasugai et al., 2010). The kinetic models for GABA binding to these receptors was taken from (Haas and Macdonald, 1999) and were corrected for temperature dependence using a $Q_{10} = 3$ (Gonzales et al., 2007), to obtain a more faithful representation of $GABA_A$ receptor activation at physiological temperature. The kinetic models of γ- and δ-subunit containing $GABA_A$ receptors are shown in **Figure 2**. A beta version of CellBlender was used to simulate repeated release events (**Figure 4**). A summary of all the simulation parameters in reported in **Table 1**.

MODEL DESCRIPTION: DIFFUSION PROPERTIES

We previously used an electron microscopy analysis to estimate the extracellular volume fraction of the mouse *stratum radiatum* hippocampal neuropil ($\alpha = 0.15$) and integrative optical imaging and two-photon laser scanning microscopy analysis to estimate the tortuosity value in this region of the brain ($\lambda = 1.45$) (Scimemi et al., 2009). The measure of λ that we obtained with this approach includes a geometric (λ_g) and a viscous component (λ_v), where $\lambda = \lambda_g \cdot \lambda_v$. The geometric component describes the hindrance to diffusion by cellular processes and by cell membrane invaginations that create dead-end routes (Hrabetova et al., 2003; Hrabetova and Nicholson, 2004; Kinney et al., 2013); the viscous component describes the hindrance to diffusion due to the presence of charged, long-chain molecules in the extracellular matrix that drag neurotransmitters as they travel in the neuropil. The relationship between λ_g and α, is:

$$\lambda_g = \sqrt{\frac{3-\alpha}{2}}$$

(see also Tao and Nicholson, 2004). In this equation, $\alpha = 0.15$ (see above) and therefore $\lambda_g = 1.19$. From the expression $\lambda = \lambda_g \cdot \lambda_v$ we estimated $\lambda_v = 1.22$. We reasoned that the viscous component of the tortuosity is the main factor that hinders neurotransmitter diffusion inside the synaptic cleft, where there are no cell process that create physical obstacles to diffusion (Barbour, 2001). The apparent diffusion coefficient (D^*) is defined as $D^* = D_{free}/\lambda^2$. We approximated the value of the apparent diffusion coefficient in the cleft (D^*_{cleft}) to$D^*_{cleft} = D_{free}/\lambda_v$, the free diffusion coefficient for GABA (D_{free}) with the free diffusion coefficient for glutamine ($D_{free} = 0.76\,\mu m^2/ms$) (Longsworth, 1953), and estimated $D^*_{cleft} = 0.51\,\mu m^2/ms$. The diffusion coefficient in the neuropil ($D^*_{neuropil}$) was estimated as $D^*_{neuropil} = D_{free}/\lambda$ (i.e., $0.36\,\mu m^2/ms$), and was in close agreement with the diffusion coefficient for glutamate derived experimentally by Nielsen et al. (2004).

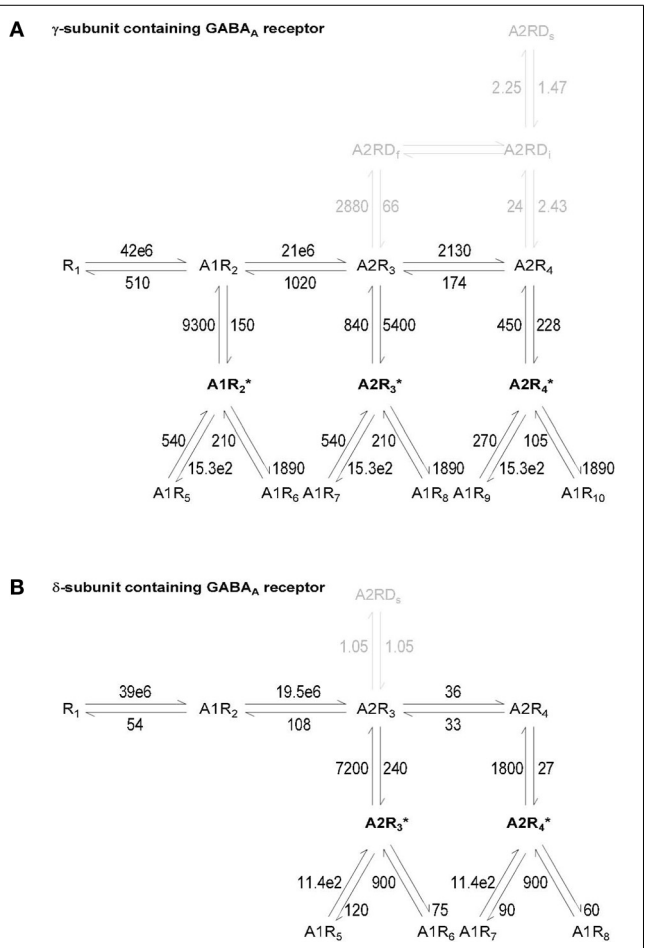

FIGURE 2 | Kinetic model of γ- and δ-subunit containing $GABA_A$ receptors. The kinetic models of γ-subunit containing $GABA_A$ receptors **(A)** and δ-subunit containing $GABA_A$ receptors **(B)** correspond to the ones developed by Haas and Macdonald (1999) and were corrected for a $Q_{10} = 3$ (Gonzales et al., 2007). Agonist molecules are indicated by A, resting states of the receptor by R, desensitized states by D and open states by an asterisk. D_f, D_i and D_s represent the fast, intermediate and slow desensitized states of γ-subunit containing $GABA_A$ receptors. The δ-subunit containing $GABA_A$ receptors only have a D_s state. The units for all rate constant are s^{-1} except for GABA binding states, which are expressed in $M^{-1}s^{-1}$.

MODEL DESCRIPTION: GABA TRANSPORTER KINETICS AND DENSITY OF EXPRESSION

The kinetics of GABA transporters (GATs) was modeled according to the following reaction scheme:

$$GAT + GABA_{out} \underset{k_{off}}{\overset{k_{on}}{\leftrightarrow}} GAT - GABA \overset{k_{cycle}}{\rightarrow} GAT + GABA_{in} \quad (1)$$

The scheme includes a rapid and reversible GABA binding step and a slow and unidirectional translocation step, analogous to the one used to simulate the activity of glutamate transporters at excitatory synapses (Barbour, 2001; Diamond, 2001, 2005; Scimemi et al., 2009). In this simplified scheme, GABA transporters do not operate in the reverse mode [i.e., they do not release GABA from

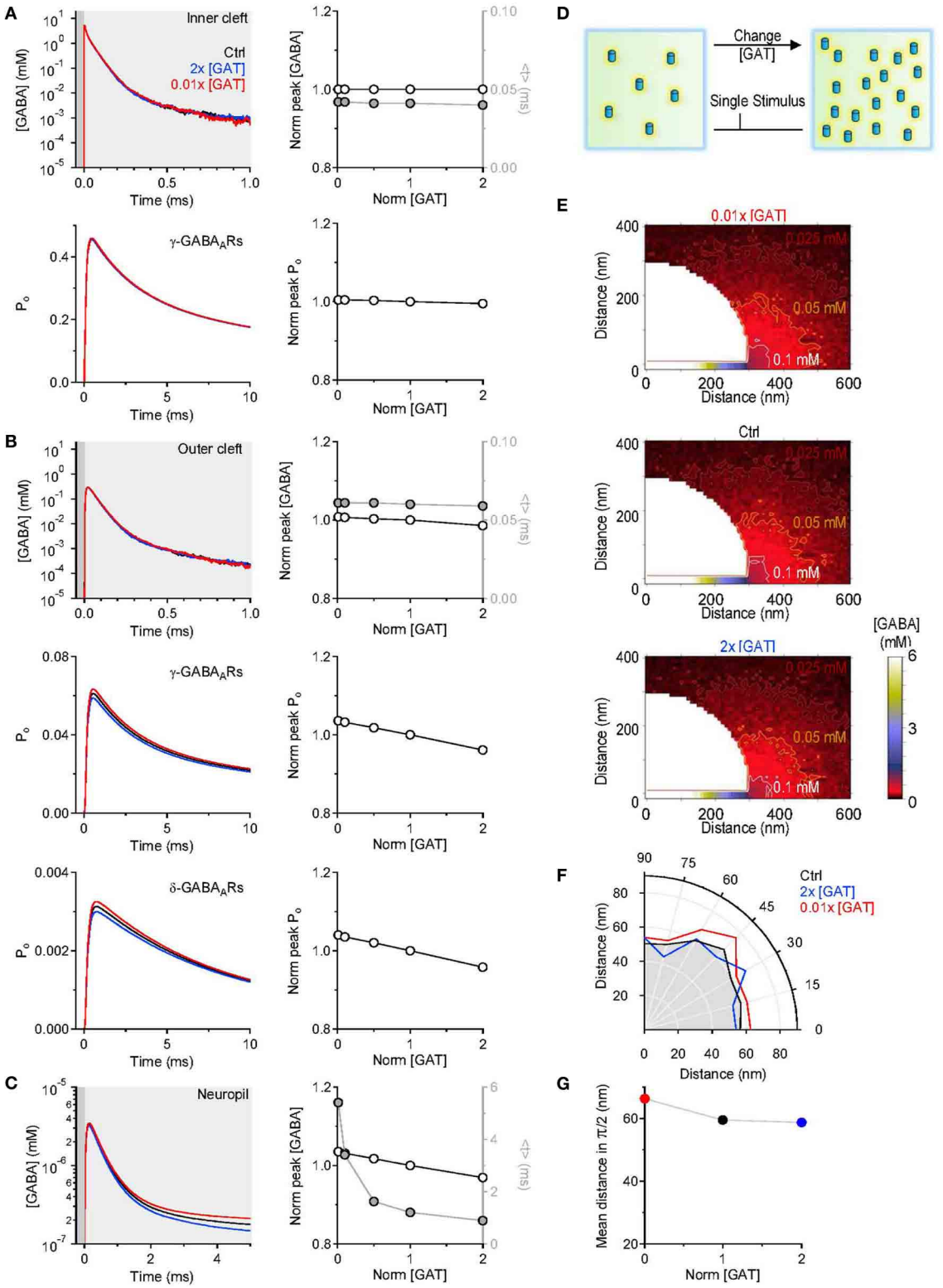

FIGURE 3 | Changing the concentration of GABA transporters alters the lifetime of GABA outside the synaptic cleft. (A) GABA concentration profile in the inner cleft volume measured when varying the concentration of GABA transporters in the entire simulation environment (top left). The peak of the GABA concentration was normalized by its value in control conditions (*left axis and white symbols*). The normalized peak and the centroid (< t >, *right axis and gray symbols*) of the GABA concentration profile in the inner cleft volume are not altered when varying the density of expression of GABA transporters (top right). No change is observed in the time peak of the open probability of γ-subunit containing $GABA_A$ receptors activated by the GABA concentration profile in the inner cleft volume (bottom right). **(B)** As in **(A)**, but

(Continued)

FIGURE 3 | Continued
the GABA concentration profile is measured in the outer cleft volume. The GABA concentration profile in the outer cleft volume is marginally influenced by changes in the density of expression of GABA transporters. This causes a progressive reduction in the activation of γ- and δ-subunit containing $GABA_A$ receptors. **(C)** GABA concentration profile in the neuropil (left). Increasing the concentration of GABA transporters in the entire simulation environment leads to a small reduction in the peak and to a pronounced decrease in the lifetime of extracellular GABA. Right: the centroid of the GABA concentration profile becomes progressively smaller at higher concentration levels of GABA transporters ($< t >$, *right axis and gray symbols*); this effect is associated with a small decrease in the peak GABA concentration (*left axis and white symbols*). **(D)** Schematic diagram illustrating the change in GABA transporter concentration performed in the simulations analyzed in this figure. Each simulation involved a single release event. **(E)** Profile of the average GABA concentration measured in the neuropil surrounding the active GABAergic pre-synaptic terminal. The panels illustrate the distribution of the mean GABA concentration observed when varying the control GABA transporter concentration (middle) from 0.01 times (top) to 2 times its value in control conditions (bottom). The white, orange, and brown contours define the regions where the GABA concentration reached values of 0.1, 0.05, and 0.025 mM, respectively. **(F)** The polar graph illustrates the change in the spatial spread of the mean GABA concentration observed when varying the concentration of GABA transporters in the entire neuropil. The lines plotted in the graph were obtained by measuring the distance between the edge of the synapse and the 0.1 mM line shown in **(E)**. **(G)** Average distance between the edge of the synaptic cleft and the 0.1 mM line shown in **(E)**, for all the 0-π/2 angles analyzed in the polar plot shown in **(F)**.

the cytosol to the extracellular space (Heja et al., 2012; Kirischuk et al., 2012)]. The rate of GABA binding to GAT1 (k_{on}) was set to $5.9{\cdot}10^6$ M^{-1} s^{-1} (Bicho and Grewer, 2005) and the unbinding rate (k_{off}) was derived using the law of conservation of mass, whereby $k_{off} = k_{on} \cdot k_m - -k_{cycle} = 58.4s^{-1}$. In this equation, k_m and k_{cycle} represent the steady-state apparent affinity for GABA ($k_m = 12.1 \cdot 10^{-6}$ M) and the turnover rate of GAT1 ($k_{cycle} = 13\,s^{-1}$), respectively (Bicho and Grewer, 2005). All rates were multiplied by $Q_{10} = 3$ to account for the temperature dependence of the reactions and describe their behavior at physiological temperature (Gonzales et al., 2007). Previous work on knock-in mice expressing GFP-tagged GAT1 has shown that the density of GAT1 expression in pre-synaptic boutons of GABAergic hippocampal interneurons is 800–1300 μm^{-2} (Chiu et al., 2002). According to this study, only 61–63% of these molecules are expressed on the plasma membrane, leading to an estimated density of expression of GAT1 on the cell membrane of pre-synaptic boutons of 496–806 μm^{-2}. In our simulations, we set the density of expression of GAT1 on the cell membrane of the pre-synaptic bouton in control conditions to $[GAT1]_{pre}$=650 μm^{-2}, which corresponds to the mid-range of the available experimental estimates. The study by Chiu et al. (2002) also indicates that the density of expression of GAT1 in the whole hippocampal neuropil is 6000 μm^{-3}. In our simulations, the control density of expression of GAT1 in the neuropil was set to $[GAT1]_{neuropil}$ = 3720 μm^{-3} (i.e., 62% of 6000 μm^{-3}). Because GAT3 is only expressed in astrocytes (Minelli et al., 1996), and because the proportion of astrocytic vs. total plasma membranes in the hippocampal neuropil is ∼10% (Lehre and Danbolt, 1998), we set the density of expression of GAT3 to 10% of that of GAT1 (i.e., $[GAT3]_{neuropil}$=372 μm^{-3}). GATs were immobile except in the simulations described in **Figure 5**, where their apparent diffusion coefficient was increased up to 2 μm^2/ms, comparable with the lateral diffusion coefficient of various neurotransmitter receptors and transporters (Heine et al., 2008; Levi et al., 2008; Bannai et al., 2009; Chamma et al., 2013).

CHANGES IN GABA TRANSPORTER EXPRESSION ALTER THE GABA CONCENTRATION PROFILE AWAY FROM THE SYNAPTIC CLEFT

In the first set of simulations, we asked how changing the density of expression of GABA transporters alters the GABA concentration profile in the volume of the inner cleft (where GABA is released; **Figure 1B** left), the outer cleft (the portion that surrounds the site of GABA release; **Figure 1B** right) and in the neuropil (the portion of the simulation environment that is not occupied by the pre-synaptic terminal and the soma; **Figure 1A** left). The concentration of GABA transporters (GATs) was varied between 0.01 and 2 times the value used to describe the concentration of GABA transporters in control conditions (Chiu et al., 2002; **Table 1**). To quantify the effects of these manipulations, we calculated the peak and the centroid of the GABA concentration profile ($< t >$). The centroid is defined as:

$$< t >= \frac{\int_{0.05F(t)_{Max}}^{0.05F(t)_{Max}} t \cdot F(t)dt}{\int_{0.05F(t)_{Max}}^{0.05F(t)_{Max}} F(t)dt}, \quad (2)$$

where $F(t)$ represents the time course of the GABA concentration profile averaged across all simulations and t represents time (see also Diamond, 2005; Scimemi et al., 2009). The centroid represents the center of mass of $F(t)$, or the average position, in time, of all the points in $F(t)$. It is calculated over a time window that corresponds to 0.05 of the peak of $F(t)$ [$F(t)_{Max}$], before and after its onset. The results presented in **Figure 3** indicate that varying the concentration of GABA transporters had no effect on the GABA concentration profile in the inner cleft volume: there was no change in the peak and in the centroid of the GABA concentration profile (**Figure 3A** top). As expected, this led to no change in the open probability (P_o) of γ-subunit containing $GABA_A$ receptors in the inner cleft area (**Figure 3A**). The effect on the GABA concentration profile in the outer cleft volume was modest, but led to a small progressive decline in the activation of γ- and δ-subunit containing $GABA_A$ receptors in this region (**Figure 3B**). When monitoring the GABA concentration profile in the neuropil, we observed a small, progressive reduction in the peak and a significant decrease in the centroid of the GABA concentration profile at higher GABA transporter concentrations (**Figure 3C**). We used these simulations to derive a spatial map of GABA diffusion from its point of release toward the surrounding neuropil (**Figure 3E**). Consistent with the previous data, lowering the expression of GABA transporters allowed GABA to diffuse further away from the active synapse (**Figures 3F,G**). These findings remained qualitatively unaltered when taking into account the presence of a tonic

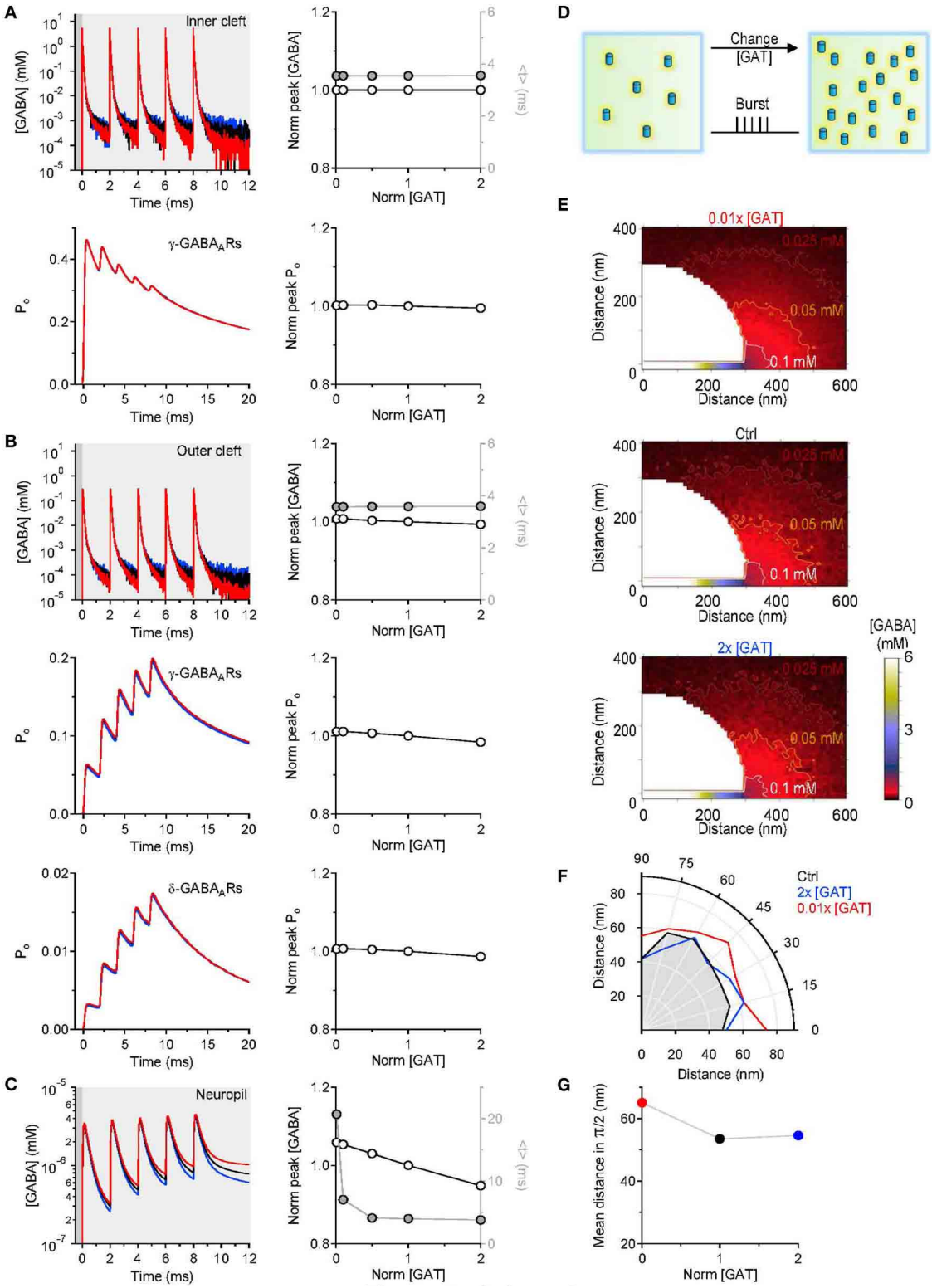

FIGURE 4 | Simulations results obtained with repeated GABA release events. (A) Five consecutive GABA release events, 2 ms apart from each other, were simulated. The GABA concentration profile in the inner cleft volume was measured when varying the concentration of GABA transporters in the entire simulation environment (top left). The peak corresponded to the maximum GABA concentration evoked by the repeated stimuli and was normalized by the value measured in control conditions. The normalized peak (*left axis and white symbols*) and the centroid (*<t>, right axis and gray symbols*) of the GABA concentration profile in the inner cleft volume are not altered when varying the density of expression of GABA transporters (top right). The open probability (P_o) of γ-subunit containing $GABA_A$ receptors

(Continued)

FIGURE 4 | Continued
in the inner cleft region declines progressively with each GABA release event. The peak of the open probability of γ-subunit containing $GABA_A$ receptors does not change when varying the concentration of GABA transporters (bottom right). **(B)** As in **A**, but the GABA concentration profile is measured in the outer cleft volume. The repeated GABA release events lead to a progressive increase in the open probability of γ-subunit containing $GABA_A$ receptors in the outer cleft area. The GABA concentration profile in the outer cleft volume is marginally influenced by changes in the density of expression of GABA transporters. This causes minor changes in the activation of γ- and δ-subunit containing $GABA_A$ receptors in the outer cleft area. **(C)** GABA concentration profile in the neuropil (left). Increasing the concentration of GABA transporters in the entire simulation environment leads to a progressive reduction in the lifetime and peak concentration of extracellular GABA. Right: the centroid of the GABA concentration profile becomes progressively smaller at higher GABA transporters concentrations ($< t >$, *right axis and gray symbols*); this effect is associated with a small decrease in the peak GABA concentration (*left axis and white symbols*). In these simulations, the centroid of the GABA concentration profile in the neuropil is calculated over a time window that corresponds to 0.20 of the peak of $F(t)$ [$F(t)_{Max}$], before and after its onset. **(D)** Schematic diagram illustrating the change in GABA transporter concentration performed in the simulations analyzed in this figure. Five release events, separated by 2 ms intervals, were simulated. **(E)** Profile of the average GABA concentration measured in the neuropil surrounding the active GABAergic pre-synaptic terminal. The panels illustrate the distribution of the mean GABA concentration observed when varying the control GABA transporter concentration (middle) from 0.01 times (top) to 2 times its value in control conditions (bottom). The white, orange and brown contours define the regions where the GABA concentration reached values of 0.1 mM, 0.05 mM and 0.025 mM, respectively. **(F)** The polar graph illustrates the change in the spatial spread of the mean GABA concentration observed when varying the concentration of GABA transporters in the entire neuropil. The lines plotted in the graph were obtained by measuring the distance between the edge of the synapse and the 0.1 mM line shown in **(E)**. **(G)** Average distance between the edge of the synaptic cleft and the 0.1 mM line shown in **(E)**, for all the 0-π/2 angles analyzed in the polar plot shown in **(F)**.

Table 1 | Parameters used for the 3D Monte Carlo reaction-diffusion simulations.

Parameter	Abbreviation	Value	References
World volume	V_{world}	1331 μm^3	
Radius of the post-synaptic soma	r_{post}	5 μm	Ascoli et al., 2008
Radius of the pre-synaptic terminal	r_{pre}	0.3 μm	Nusser et al., 1997; Specht et al., 2013; cf. Schikorski and Stevens, 1997
Radius of the inner cleft (i.e., radius of the active zone and of the inhibitory post-synaptic density)	r_{icleft}	0.1 μm	Biro et al., 2006; Specht et al., 2013; cf. Kasugai et al., 2010
Radius of the outer cleft (i.e., radius of the peri-synaptic annulus)	r_{ocleft}	0.2 μm	Nusser et al., 1997; cf. Schikorski and Stevens, 1997
Number of GABA molecules released	n_{GABA}	2000	
Extracellular volume fraction	α	0.15	Scimemi et al., 2009
Tortuosity	λ	1.45	Scimemi et al., 2009
Free GABA diffusion coefficient (cf. glutamine)	D_{free}	0.76 μm^2/ms	Longsworth, 1953
Apparent GABA diffusion coefficient in the cleft	D^*_{cleft}	0.51 μm^2/ms	
Apparent GABA diffusion coefficient in the neuropil	D^*_{world}	0.36 μm^2/ms	
GAT binding rate	k_{on}	$5.9 \cdot 10^6$ $M^{-1}s^{-1}$	Bicho and Grewer, 2005
GAT unbinding rate	k_{off}	58.4 s^{-1}	
GAT steady-state affinity	k_m	$12.1 \cdot 10^{-6}$ M	Bicho and Grewer, 2005
GAT turnover rate	k_{cycle}	13 s^{-1}	Bicho and Grewer, 2005
GAT temperature dependence	Q_{10}	3	Gonzales et al., 2007
GAT1 density in the pre-synaptic terminal	$[GAT1]_{pre}$	650 μm^{-2}	Chiu et al., 2002
GAT1 density in the neuropil	$[GAT1]_{neuropil}$	3720 μm^{-1}	Chiu et al., 2002
GAT3 density in the neuropil	$[GAT3]_{neuropil}$	372 μm^{-3}	
Simulations time step	Δt	1–10 μs	
Simulations iterations	n_{iter}	5000–50,000	
Simulations seeds	n_{seed}	30–100	

extracellular GABA concentration of 160 nM (Santhakumar et al., 2006) (data not shown). This is probably not surprising, because this concentration is significantly lower than the substrate steady-state affinity of GABA transporters for GABA (Bicho and Grewer, 2005). The data indicate that the main effects of altering the expression of GABA transporters are detected at a distance from an active synapse. It is the activation of GABAreceptors away from the release site—not of those directly opposed to it—that can be regulated by changing the density of expression of GABA transporters. The proportion of GABA molecules that can be bound by GABA transporters in the cleft is small. This is consistent with the notion that the activation of receptors in the immediate vicinity

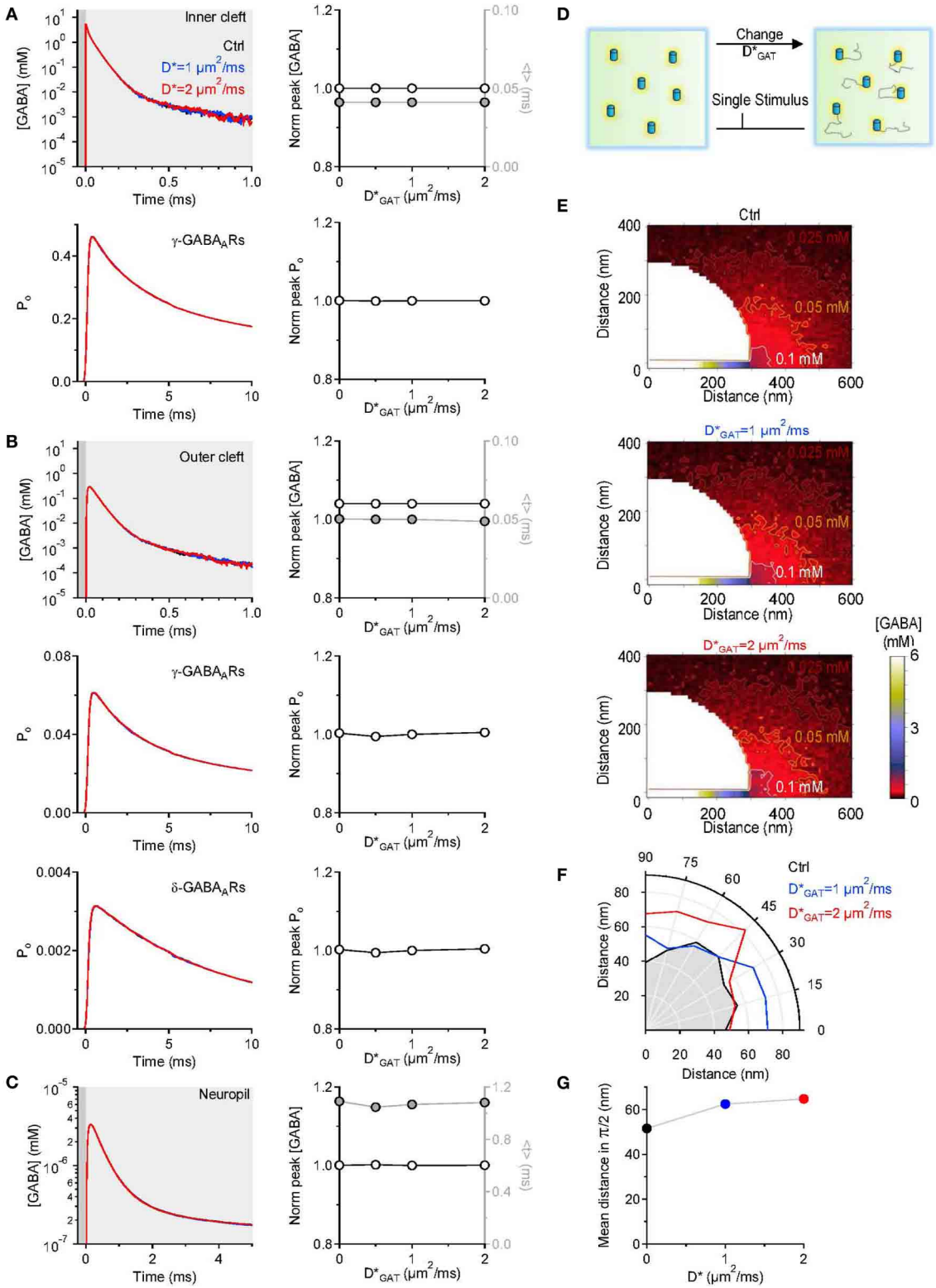

FIGURE 5 | Changing the lateral mobility of GABA transporters alters spatial spread but not the lifetime of GABA outside the synaptic cleft. **(A)** GABA concentration profile in the inner cleft volume measured when varying the lateral mobility of GABA transporters in the entire simulation environment (top left). The centroid (< t >) of the GABA concentration profile in the inner cleft volume is not altered when varying the lateral mobility of GABA transporters (top right). No change is observed in the time course (bottom left) and peak (bottom right) of the open probability of γ-subunit containing $GABA_A$ receptors activated by the GABA concentration profile in

(Continued)

FIGURE 5 | Continued
the inner cleft volume. **(B)** As in **(A)**, but the GABA concentration profile is measured in the outer cleft volume. The GABA concentration profile in the outer cleft volume is not influenced by changes in the lateral mobility of GABA transporters. This leads to no change in the activation of γ- and δ-subunit containing $GABA_A$ receptors. **(C)** GABA concentration profile in the neuropil (left). Increasing the lateral mobility of GABA transporters in the entire simulation environment does not alter the lifetime of GABA in the extracellular space. The peak concentration (*left axis and white symbols*) and the centroid of the GABA concentration profile (*right axis and gray symbols*) are not affected by increasing the lateral mobility of GABA transporters (right). **(D)** Schematic diagram illustrating the change in GABA transporter apparent diffusion coefficient (D^*_{GAT}) performed in the simulations analyzed in this figure. **(E)** Profile of the average GABA concentration measured in the neuropil surrounding the active GABAergic pre-synaptic terminal. The panels illustrate the distribution of the mean GABA concentration observed when varying the GABA transporter lateral mobility from 0 (top) to 1 μm^2/ms (middle) and 2 μm^2/ms (bottom). The white, orange and brown contours define the regions where the GABA concentration reached values of 0.1, 0.05, and 0.025 mM, respectively. **(F)** The polar graph illustrates the change in the spatial spread of the mean GABA concentration observed when varying the lateral mobility of GABA transporters in the entire neuropil. The lines plotted in the graph were obtained by measuring the distance between the edge of the synapse and the 0.1 mM line shown in **(E)**. **(G)** Average distance between the edge of the synaptic cleft and the 0.1 mM line shown in **(E)**, for all the 0-π/2 angles analyzed in the polar plot shown in **(F)**.

of an active release site is dominated by diffusion, not by the activity of transporters (Rusakov and Kullmann, 1998; Barbour, 2001; Scimemi and Beato, 2009; Scimemi et al., 2009). Notably, the notion holds even at GABAergic synapses, where the neurotransmitter transporters are expressed also in the synaptic cleft region (not only at the edge of it as it happens for glutamatergic synapses He et al., 2000).

GABAergic interneurons in the hippocampus can fire bursts of action potentials and in some cases each action potential can evoke the release a synaptic vesicle (Freund and Buzsaki, 1996). We asked whether the results of our simulations would be different when multiple GABA release events are triggered from the pre-synaptic active zone. To address this, we simulated a burst of five release events, 2 ms apart from each other (**Figure 4**). Each time, 2000 GABA molecules were released from the center of the synapse in the synaptic cleft. Even in this case, varying the density of expression of GABA transporters did not induce any significant change in the peak and time course of the GABA concentration profile in the inner cleft volume (**Figure 4A** top). The peak GABA concentration in the inner cleft volume increased to ~5.3 mM with each release event. This caused progressive desensitization of γ-subunit containing $GABA_A$ receptors in the inner cleft region, at all tested levels of GABA transporter expression (**Figure 4A** bottom). The GABA concentration profile in the outer cleft region was also not significantly affected by changes in the GABA transporter concentration (**Figure 4B**). Here, the activation of γ- and δ-subunit containing $GABA_A$ receptors increased progressively with consecutive release events, and was insensitive to changes in GABA transporter concentration (**Figure 4B**). Similarly to what observed with single stimulations, increasing the expression of GABA transporters caused a small reduction in the peak and a profound decrease in the lifetime of extracellular GABA (**Figure 4C**). Consistent with these findings, the spatial maps of GABA diffusion showed that GABA diffused at higher concentration, further away from the release site when lowering the expression of GABA transporters (**Figures 4E–G**). A GABA transporter density of expression of 650 μm^{-2} (Chiu et al., 2002) and a pre-synaptic apposition area of 0.28 μm^2 (**Table 1**) result in the presence of 182 GABA transporter molecules in the pre-synaptic membrane within the cleft region. The majority of the synaptically-released GABA molecules diffuse away from the synaptic cleft before they are bound by the transporters. Therefore, GABAergic transmission mediate by receptors located at the center of the synapse is preserved regardless of the expression levels of GABA transporters.

CHANGES IN GABA TRANSPORTER LATERAL MOBILITY ALTERS THE SPATIAL SPREAD, NOT THE LIFETIME OF GABA OUTSIDE THE SYNAPTIC CLEFT

In the simulations described above, we assumed GABA transporters to be completely immobile within the cell membrane. There is experimental evidence that indicates that there are adapter proteins, like ezrin, that anchor GABA transporters in the plasma membrane to the cell cytoskeleton (Imoukhuede et al., 2009; Moss et al., 2009). The proportion of immobile GABA transporters represents 50% of the entire population of surface GABA transporters (Imoukhuede et al., 2009; Moss et al., 2009). At steady-state, an increase in the lateral mobility of GABA transporters is associated with increased GABA uptake (Imoukhuede et al., 2009). Synaptic transmission is not a steady-state event, and the functional implications of changes in the mobility of GABA transporters are incompletely understood. To resolve this issue, in a separate set of simulations, we varied the diffusion coefficient of GABA transporters within the cell membrane and tested the effect that this had on the GABA concentration profile at different distances from an active release site. The diffusion coefficient was increased from 0 to 2 μm^2/ms, a value that is consistent with the estimated diffusion coefficient of other neurotransmitter transporters and receptors (Heine et al., 2008; Levi et al., 2008; Bannai et al., 2009; Chamma et al., 2013). We found that increasing the lateral mobility of GABA transporters did not affect the peak and time course of the GABA concentration profile and the activation of GABA receptors in the inner (**Figure 5A**) and outer cleft (**Figure 5B**). The lifetime of GABA in the neuropil was also unaltered (**Figure 5C**). The only effect that we could detect was that GABA diffused further away from its release site when GABA transporters were mobile (**Figures 5E–G**). Therefore, increasing the mobility or the proportion of mobile surface GABA transporters facilitates GABA diffusion away from the synaptic cleft. This effect is likely to become more pronounced if the mobile GABA transporters have: (1) rapid binding and slow unbinding kinetics; (2) high lateral diffusion coefficient; (3) low transport efficiency. Under these conditions, the lifetime of the GABA-bound state would be longer than the time required for the lateral diffusion of GABA transporters away from the synaptic cleft and this could contribute to degrade the spatial specificity of GABAergic synaptic transmission.

CONCLUSIONS

GABA transporters are expressed in neurons and astrocytes, but in most regions of the brain they reach the highest levels of expression in neuronal pre-synaptic terminals (Zhou and Danbolt, 2013). The naïve intuition is that this location is perfectly tailored to remove GABA from the synaptic cleft immediately after its release, allowing for rapid neurotransmitter recycling into pre-synaptic terminals during repeated stimulations. To date, it has not been tested whether this hypothesis holds given the rapid kinetics of neurotransmitter diffusion in the extracellular space and the binding and translocation kinetics of GABA transporters (Bicho and Grewer, 2005). Here we used a series of 3D Monte Carlo reaction-diffusion simulations to determine the effect of varying GABA transporter density of expression and surface mobility on the GABA concentration profile and the recruitment of GABA receptors at different distances from an active release site. Our findings indicate that altering surface expression and mobility of GABA transporters do not lead to changes in the GABA concentration profile in the inner portion of the synaptic cleft. In contrast, the lifetime of GABA in the neuropil surrounding an active GABAergic synapse is prolonged by reducing the density of expression of GABA transporters. Increasing the lateral mobility of GABA transporters facilitates GABA diffusion away from the synaptic cleft. These results are conceptually important because they indicate that the activation of synaptic receptors is not affected by the presence of synaptic GABA transporters. The activity-dependent regulation of intracellular signaling cascades that control the surface expression and mobility of GABA transporters mainly affect the recruitment of extrasynaptic—not synaptic—GABA receptors. Therefore, PKC and tyrosine kinase, which control the cytoplasm/cell membrane partitioning of GABA transporters, cam modify the spatial spread of GABAergic signals. These findings suggest novel mechanisms to tune the plasticity and spatial specificity of GABAergic synaptic transmission in the brain.

ACKNOWLEDGMENTS

Thanks to J. Czech and M. Dittrich for support with MCell and CellBlender; N. C. Danbolt for valuable discussions; M. Beato, P. E. Forni, S. Bellini, and A. M.Sinensky for valuable comments on the manuscript. This work was supported by the Departmental Start-up Grant and by the Research Foundation of SUNY Albany.

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

Received: 03 March 2014; accepted: 22 April 2014; published online: 13 May 2014.
*Citation: Scimemi A (2014) Plasticity of GABA transporters: an unconventional route to shape inhibitory synaptic transmission. Front. Cell. Neurosci. **8**:128. doi: 10.3389/fncel.2014.00128*
This article was submitted to the journal Frontiers in Cellular Neuroscience.

frontiers in
CELLULAR NEUROSCIENCE

REVIEW ARTICLE
published: 09 April 2014
doi: 10.3389/fncel.2014.00103

Gephyrin phosphorylation in the functional organization and plasticity of GABAergic synapses

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Gephyrin is a multifunctional scaffold protein essential for accumulation of inhibitory glycine and $GABA_A$ receptors at post-synaptic sites. The molecular events involved in gephyrin-dependent $GABA_A$ receptor clustering are still unclear. Evidence has been recently provided that gephyrin phosphorylation plays a key role in these processes. Gephyrin post-translational modifications have been shown to influence the structural remodeling of GABAergic synapses and synaptic plasticity by acting on post-synaptic scaffolding properties as well as stability. In addition, gephyrin phosphorylation and the subsequent phosphorylation-dependent recruitment of the chaperone molecule Pin1 provide a mechanism for the regulation of GABAergic signaling. Extensively characterized as pivotal enzyme controlling cell proliferation and differentiation, the prolyl-isomerase activity of Pin1 has been shown to regulate protein synthesis necessary to sustain the late phase of long-term potentiation at excitatory synapses, which suggests its involvement at synaptic sites. In this review we summarize the current state of knowledge of the signaling pathways responsible for gephyrin post-translational modifications. We will also outline future lines of research that might contribute to a better understanding of molecular mechanisms by which gephyrin regulates synaptic plasticity at GABAergic synapses.

Keywords: gephyrin, phosphorylation, $GABA_A$ receptors, GSK-3β signaling, ERK signaling, Pin1

INTRODUCTION

Post-synaptic scaffolding molecules are key factors for the functional organization of synapses. They ensure the accurate accumulation of neurotransmitter receptors in precise apposition to pre-synaptic release sites as required for a reliable synaptic transmission. Scaffolding molecules also interact with cytoskeletal anchoring elements and these interactions are thought not only to provide the physical constraints for maintaining receptors at synapses, but also for regulating the constant flux of receptors and scaffolding elements in and out of post-synaptic sites (Choquet and Triller, 2003; Hanus et al., 2006). They can also regulate downstream signaling pathways to adjust the molecular composition of the post-synaptic devices necessary to sustain synaptic plasticity. At inhibitory post-synaptic densities (PSDs) a single protein, gephyrin, builds the major scaffold for the transient immobilization of inhibitory glycine receptors (GlyRs) and α2-γ2 subunits containing $GABA_A$ receptors ($GABA_A$Rs; Tretter et al., 2012). The formation and maintenance of gephyrin clusters rely mostly on gephyrin-gephyrin interactions (reviewed in Fritschy et al., 2008). Gephyrin is a 93-kDa protein that consists of three major domains: an N-terminal G-domain, a C-terminal E-domain and a connecting central linker region (C-domain) (Prior et al., 1992). Crystal structure studies have demonstrated that while the G-domain has an intrinsic tendency to trimerize the E-domain dimerizes (Schwarz et al., 2001; Sola et al., 2001, 2004). These oligomerization features suggest a model for cluster formation whereby gephyrin builds a bidimensional hexagonal lattice underneath the synaptic membrane (Kneussel and Betz, 2000; Schwarz et al., 2001; Sola et al., 2001, 2004; Xiang et al., 2001) which exposes a high number of binding sites for GlyR β subunits and for $GABA_A$Rs α1, α2, α3, β2 and β3 subunits (Maric et al., 2011; Kowalczyk et al., 2013).

Recently, an elegant study based on quantitative three-dimensional nanoscopic imaging, has not only confirmed that gephyrin clusters are indeed bidimensional planar structures lying underneath the synaptic plasma membrane but has also provided evidence that all gephyrin molecules in the cluster are potentially capable to interact with neurotransmitter receptors localized in the synaptic membrane in a stoichiometry ratio gephyrin-receptor of approximately 1:1 (Specht et al., 2013).

A consequence of this organization is that changes in gephyrin clustering could produce parallel changes in the number of receptors trapped by the scaffold, and thus lead to corresponding alteration of the strength of synaptic transmission. This may vary with age and in different cell compartments as suggested by the transient expression of gephyrin clusters co-localized with $GABA_A$Rs at immature perisomatic but not dendritic basket-Purkinje cell synapses (Viltono et al., 2008). The loss of gephyrin and the consequent re-organization of perisomatic $GABA_A$R clusters in more mature neurons may affect their trafficking and stability.

Another important element in the functional organization of inhibitory synapses is represented by the affinity of gephyrin for neurotransmitter receptors (Fritschy et al., 2008). Mechanisms that are able to alter these parameters could uncouple gephyrin clustering and the number of receptors that can be effectively accommodated within the cluster itself. This mechanism would be well suited for the complex and still poorly understood dynamics of gephyrin-dependent $GABA_A$Rs (Tretter et al., 2012). In contrast to GlyRs that interact with gephyrin only through the β subunits, $GABA_A$Rs interact *via* their large intracellular loops with several subunits of the α and β families such α1, α2, α3 and β2, β3, respectively (Tretter et al., 2008, 2011; Saiepour et al., 2010; Mukherjee et al., 2011; Kowalczyk et al., 2013). These subunits utilize the same binding site as GlyR (Maric et al., 2011) but display a binding affinity at least one order of magnitude lower. The γ2 subunit, initially thought to be implicated in controlling gephyrin-dependent $GABA_A$Rs clustering (Essrich et al., 1998), as its gene deletion strongly affects both receptor and gephyrin synaptic accumutation (Günther et al., 1995), was never identified as direct interactor of gephyrin (Tretter et al., 2012). The α4, α5 and δ subunits present mainly on extrasynaptic $GABA_A$Rs lack of co-localization with gephyrin (Farrant and Nusser, 2005). While each $GABA_A$R is a pentamer, it is still not known which available binding sites are actively involved in gephyrin interaction and whether and how they cooperate to increase the overall binding affinity for gephyrin. Finally, gephyrin dynamics rely on its availability for cluster formation which depends on its regulated transport to post-synaptic sites and degradation. Degradation requires mainly the activity of the Ca^{2+}-dependent cysteine protease calpain-1 (Kawasaki et al., 1997; Tyagarajan et al., 2011).

The recruitment of gephyrin to GABAergic synapses needs the contribution of at least two classes of interactors: the cell adhesion molecules of the neuroligin (NL) family (Südhof, 2008) and the guanine nucleotide exchange factor for the monomeric GTPase Cdc42 collybistin (Kins et al., 2000). In particular NL2, the isoform constitutively localized at inhibitory GABAergic synapses (Varoqueaux et al., 2004), interacts with both gephyrin and collybistin forming a ternary complex able to activate collybistin-driven gephyrin tethering to the plasma membrane followed by receptors recruitment (Poulopoulos et al., 2009).

In summary, several gephyrin-dependent mechanisms affect the number of $GABA_A$Rs at synaptic sites at any given time, and thereby may influence the strength of synaptic transmission: gephyrin-gephyrin interaction, gephyrin-receptor (neurotransmitters or other synaptically localized membrane proteins) binding affinities, gephyrin turnover and synaptic transport. Recently new mechanistic insights on the regulation of gephyrin oligomerization, stability and receptor binding capability have been provided. They suggest that phosphorylation, (a versatile mechanism for regulating protein activity in a specific and controlled manner), already involved in the functional modulation of receptors at synapses, is determinant for all aspect of gephyrin dynamics. Interestingly, the signaling pathways altering the phosphorylation status of gephyrin have been previously identified as modulator of glutamatergic signaling. The functional cross-talk between excitatory and inhibitory transmission may have important implications for the long-term stability of neuronal networks.

SIGNALING PATHWAYS INVOLVED IN GEPHYRIN CLUSTERING

A recent genome-wide siRNA screening aimed at identifying protein kinases stabilizing gephyrin clustering revealed a contribution of Receptor Tyrosine Kinases (RTKs) signaling; in particular the tropomyosin-related kinase B (Trk-B) and its ligand the brain-derived neurotrophic factor (BDNF; Wuchter et al., 2012). The BDNF-TrkB system is required for multiple aspects of neuronal functions including neuronal survival and differentiation during development as well as synaptic plasticity of mature neurons (Thoenen et al., 1987; Tanaka et al., 2000; Poo, 2001). The activation of TrkB by BDNF triggers various signaling cascades including the Ras/mitogen-activated protein (MAP) kinase (Ras/MAPK) pathway, the phosphatidylinositol 3-kinase (PI3-Kinase)/Akt pathway and the phospholipase C gamma (PLCγ) pathway (Arévalo and Wu, 2006). At glutamatergic synapses, the activation of MAPK and PI3K pathways plays a crucial role in synaptic plasticity. This occurs not only *via de novo* regulation of protein synthesis but also *via* trafficking of pre-existing synaptic proteins. Therefore, it is not surprising that these signaling pathways contribute to regulate gephyrin transport at synapses (**Figure 1**). The BDNF-dependent activation of the PI3K/Akt pathway leads to the activation of rapamycin (mTOR), a regulator of mRNA translation (Sarbassov et al., 2005). Sabatini et al. (1999) demonstrated that mTOR interacts with gephyrin and this interaction is fundamental for mTOR-dependent signaling to the translational repressor 4E-BP1 (Sabatini et al., 1999). Upon BDNF treatment mTOR decreases its association with gephyrin, thus releasing gephyrin for membrane transport and cluster assembly. In addition PI3K activation, by promoting an increase in phosphatidylinositol (3,4,5)-triphosphate (PIP3) membrane content, may enhance collybistin-mediated gephyrin recruitment at GABAergic synapses (Reddy-Alla et al., 2010). In parallel, BDNF-dependent activation of Akt was shown to promote the inactivation of the serine/threonine kinase glycogen synthase kinase 3β (GSK-3β), a recently indentified negative regulator of gephyrin clustering (Tyagarajan et al., 2011). The authors of the wide-genome screening (Wuchter et al., 2012) also provided evidence for a contribution of the MAPK signaling cascade to gephyrin clustering, independent of mTOR activation, and controlled by the negative regulators of RTKs signaling sprouty proteins (Kim and Bar-Sagi, 2004).

The screening identified two siRNA directed against testicular protein kinase 1 (Tesk1) and Dual specificity tyrosine-phosphorylation-regulated kinase 1A (Dyrk1A), two protein kinases implicated in the inhibitory phosphorylation of sprouty proteins, in particular sprouty2, that specifically inhibit the Ras-Raf-MAPK pathway triggered by BDNF (Aranda et al., 2008; Chandramouli et al., 2008). This study, while revealing mechanisms involved in the control of gephyrin clustering, did not address the possibility that such signaling cascade may also affect gephyrin phosphorylation. Tyagarajan et al. (2013) were able to demonstrate that some of the kinases belonging to the MAPK and

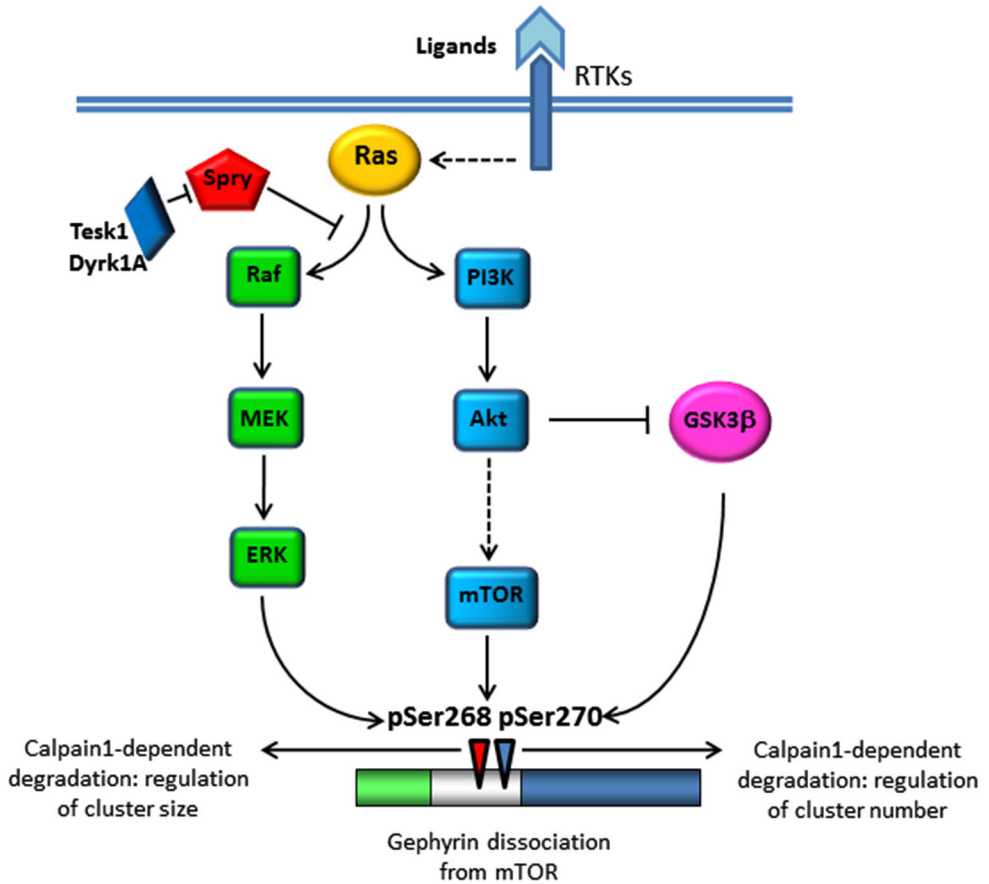

FIGURE 1 | Schematic representation of signaling pathways affecting gephyrin clustering. Stimulation of RTKs by ligand binding or activity-dependent increase in calcium levels activates Ras and its downstream signaling cascades Ras/MAPK and PI3K/Akt leading to gephyrin phosphorylation at Ser268 by ERK and gephyrin dissociation from mTOR. Akt also inhibits GSK-3β activity, the kinase responsible of Ser270 phosphorylation. Gephyrin phosphorylated by these two kinases becomes substrate of calcium-dependent calpain degradation.

PI3K/Akt signaling pathways influence gephyrin dynamics and GABAergic transmission right through direct gephyrin phosphorylation (see below).

PHOSPHORYLATION OF GEPHYRIN C-DOMAIN ALTERS ITS OLIGOMERIZATION AND STABILITY PROPERTIES

Gephyrin has been known to be a phosphoprotein since 1992, when Langosh and colleagues discovered that this protein co-purified with GlyR preparations has a kinase activity capable of promoting the incorporation of phosphate groups into serine and threonine residues (Langosch et al., 1992). The functional relevance of these post-translational modifications was neglected for long time, possibly because gephyrin was considered to be just a mere tubulin-binding protein, therefore a simple structural component of the inhibitory PSD.

Mass spectrometry analysis performed on gephyrin isolated from either mouse or rat brain homogenates or purified upon its overexpression in eukaryotic cells, has identified 22 phosphorylation sites, all located within the C-domain of gephyrin, except the threonine 324 (Thr324) site that lies in the C-terminal E-domain (**Figure 2**; Herweg and Schwarz, 2012; Kuhse et al., 2012; Tyagarajan et al., 2013). The C-domain is positioned between the highly conserved G- and E-domains that are directly involved in gephyrin multimerization. Based on its sensitivity to proteolytic cleavage (Schrader et al., 2004), the C-domain is the most exposed to the surrounding environment, making it a suitable substrate for post-traslational modifications. This domain also mediates the phosphorylation-dependent recruitment of the peptidyl prolyl *cis-trans* isomerase Pin1 (discussed below) (Zita et al., 2007), the interaction with dynein light chain (Fuhrmann et al., 2002) and contributes to the recruitment of collybistin (Zacchi et al., personal communication).

In this region, conformational changes induced by phosphorylation could affect the folding of the C-domain itself and of the neighboring G- and E-domains, thus altering gephyrin clustering properties. A recent study (Herweg and Schwarz, 2012) has demonstrated that gephyrin, once expressed in a system that allows post-translational modifications, behaves quite differently in terms of oligomerization, folding stability and receptor binding. Gephryn expressed in Spodoptera frugiperda (Sf9) insect cells shows a diffuse distribution in the cytosol instead of the characteristic "aggregates" observed in HEK293 (Meier et al.,

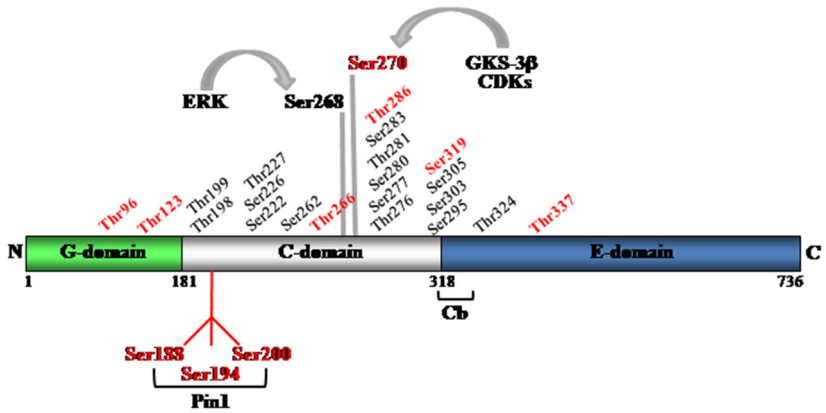

FIGURE 2 | Schematic representation of gephyrin domains and the identified phosphorylation sites. Mass spectrometry has allowed identifying 22 serine and threonine residues within the C-domain and one (threonine 324), in the E-domain. In red are highlighted all putative Pin1 consensus motifs. Ser270 and Ser268 are recognized targets of GSK-3β and ERK kinase activities, respectively.

2000) or COS7 cells (Kirsch and Betz, 1995). The basic building blocks are formed by hexamers instead of trimers; in addition, G- and C-domains form a complex with increased overall stability while E-domains are stabilized upon receptor interaction. These parameters are also sensitive to changes in the amino acid sequence of gephyrin due to alternative splicing of the gene that, interestingly, impacts mostly on its C-domain organization, further underlying the contribution of this region in determining gephyrin folding and clustering (Herweg and Schwarz, 2012). It is therefore not surprising that most of the signaling pathways able to affect gephyrin clustering are represented by serine/threonine kinases targeting specific residues embedded in the C-domain of the protein.

PHOSPHORYLATION OF GEPHYRIN AT SERINE 270 IS AT THE CROSS-ROAD OF DIFFERENT SIGNALING PATHWAYS

One of the first gephyrin residues identified as the target of specific kinases was serine 270 (Ser270; Tyagarajan et al., 2011). Interestingly, the first kinase found to promote post-translational modifications was a serine/threonine kinase belonging to the family of Glycogen Synthase Kinase 3 (GSK3), enzymes originally identified as key regulators of glucose metabolism (Woodgett and Cohen, 1984; Wang and Roach, 1993). GSK3 signaling cascades have clearly recognized roles in neurodevelopmental processes such as neurogenesis, neuronal migration, neuronal polarization and axonal growth and guidance (reviewed in Hur and Zhou, 2010). Recently they have been implicated in N-methyl-D-aspartate receptors (NMDARs)-dependent long-term depression at glutamatergic synapses (Bradley et al., 2012). Even though the underlying molecular mechanisms are still not understood, GSK-3β–dependent phosphorylation of PSD-95, the major scaffold protein of excitatory PSD, functionally homologue of gephyrin, was found to destabilize the scaffold molecule thus allowing AMPA receptors internalization and LTD induction (Nelson et al., 2013).

Like PSD-95, GSK-3β appears to exert a negative effect on gephyrin clustering at GABAergic synapses (Tyagarajan et al., 2011). Several lines of evidence support this notion. Overexpression of a gephyrin phosphodeficient mutant (Ser270Ala) in cultured hippocampal neurons promotes the formation of supernumerary gephyrin clusters similar in size to those obtained upon wild-type gephyrin overexpression. Functionally, alanine mutation at this site selectively enhances the frequency of miniature inhibitory post-synaptic currents (mIPSC), a result which is in line with the increased density of functional GABAergic synapses. Additionally, a similar phenotype was observed upon pharmacological inhibition of GSK-3β activity both *in vitro* and *in vivo*. The authors of this study also provided mechanistic insights on how GSK-3β dependent phosphorylation of Ser270 can negatively regulate gephyrin clustering. They were able to demonstrate that phosphorylated gephyrin becomes substrate of the Ca^{2+}-dependent protease calpain-1, possibly because at this location the phosphorylation-dependent conformational change may expose the sequence rich in proline (P), glutamic acid (E), serine (S) and threonine (T) (PEST sequence; Rechsteiner, 1990) that acts as a signal peptide for protein degradation. It is interesting to note that Ser270 lies also within a putative Pin1 consensus motif, raising the intriguing possibility that prolyl-isomerase may also participate in the conformational changes required to drive gephyrin proteolytic degradation. Since rises in calcium and GSK-3β activation are coupled to neuronal activity, the identified mechanisms are well suited to mediate plasticity-related changes at GABAergic synapses. Several issues remain to be unraveled regarding the functional consequences of this phosphorylation event. It will be interesting to understand how Ser270 phosphorylation destabilizes gephyrin assembled into a crowded lattice, where gephyrin is engaged in several protein-protein interactions with itself, neurotransmitter receptors and other transmembrane proteins (e.g., NL2). All these interactions represent potential targets of the signaling cascade. The fact that gephyrin phosphodeficient mutants possess synaptogenic activity further supports the notion that this site may regulate gephyrin binding to proteins important for building and maintaining functional GABAergic synapses. By converging on both scaffold

molecules PSD-95 and gephyrin, GSK-3β signaling cascade, coordinates changes at both glutamatergic and GABAergic synapses, thus allowing to maintain an appropriate excitatory/inhibitory (E/I) balance.

The picture became even more complicated by the discovery that other kinases of the CDK family, in particular Cyclin-dependent kinase 5 (Cdk5), can target the same site, making this residue at the cross-road of different signaling pathways (Kuhse et al., 2012). Cdk5 is a proline-directed serine/threonine kinase with high activity in the central nervous system. Based on sequence homology, Cdk5 belongs to a class of kinases operating in the cell cycle, even though it is not activated by traditional cyclins and it plays critical roles in several aspect of brain development and neuronal functions including neuronal migration, differentiation, synapse development and plasticity (Lai and Ip, 2009; Su and Tsai, 2011).

The precise role of Cdk5 in activity-dependent synaptic plasticity is still not understood but the identification of novel substrates and interacting molecules has provided significant mechanistic insights. At glutamatergic synapses, Cdk5 has been shown to affect NMDA receptors-dependent plasticity through several mechanisms: (i) by altering NMDA receptor channel conductance upon Cdk5-depenent phosphorylation of certain receptor subunits (Li et al., 2001); (ii) by down-regulating in an activity-dependent manner NMDA receptors number *via* a calpain-dependent proteolytic degradation (Hawasli et al., 2007); and (iii) by regulating the endocytosis of NMDA receptor *via* phosphorylation of the scaffolding molecule PSD-95 (Morabito, 2004; Zhang et al., 2008).

Members of Cdk family, in particular Cdk5, contribute to gephyrin posphorylation at Ser270. Interestingly, this event seems to be tightly controlled by the level of expression of collybistin, being its down-regulation associated with a loss of gephyrin immunoreactivity as detected by the widely used monoclonal antibody mAb7a (Kuhse et al., 2012). The authors of this study showed that the antibody mAb7a is sensitive to gephyrin phosphorylation at that specific amino acid residue, making it a *bona fide* phospho-Ser270-specific monoclonal antibody. Therefore, the observed drastic reduction of mAb7a immunoreactivity observed upon collybistin knock-down or pharmacological inhibition of CDKs in cultured hippocampal neurons, indicated a reduction in gephyrin phosphorylation at Ser270 not necessarily associated with loss of synaptic gephyrin puncta. Experiments performed by using another gephyrin-specific antibody, not sensitive to its phosphorylation status, indeed demonstrated that the number and size of gephyrin clusters were not significantly affected by these treatments. Based on these results, in a mature cluster, gephyrin is expected to be constitutively phosphorylated at position 270, detectable by the mAb7a antibody, and to undergo selective dephosphorylation upon collybistin down-regulation. In contrast, results obtained from the characterization of GSK-3β dependent phosphorylation of gephyrin support an opposite scenario. Gephyrin assembled into a cluster is expected to be mainly dephosphorylated and to undergo activity-dependent GSK-3β mediated phosphorylation to promote its proteolytic degradation followed by cluster disassembly (Tyagarajan et al., 2011). Several speculations can be put forward to place these conflicting results in a more coherent picture. One possibility is that gephyrin builds different types of clusters, the one detected by mAb7a being characterized by high turnover rates. Alternatively, gephyrin scaffold is heterogenous in respect to gephyrin modifications and that phosphorylation at Ser270, as well as at neighboring positions, may generally act by restricting gephyrin oligomerization potential.

A question raised by these findings is how collybistin exerts its regulatory effect on Cdk5-dependent gephyrin phosphorylation. Collybistin is a key interactor of gephyrin known to participate in its membrane recruitment and synaptic targeting (Papadopoulos and Soykan, 2011). This activity relies on the presence of a Pleckstrin homology domain in collybistin sequence, a domain thought to mediate the attachment of the molecule to the membrane by binding to phosphoinositides (Hyvönen et al., 1995). Most collybistin isoforms expressed in neurons possesses at their N-terminus an SH3 regulatory domain that prevents their membrane-targeting function (Kins et al., 2000; Harvey et al., 2004). At GABAergic synapses only the cell adhesion molecule NL2 (Poulopoulos et al., 2009) and the α2 subunit of $GABA_A$Rs (Saiepour et al., 2010) are capable of relieving such SH3-mediated inhibition, possibly by binding to it, thus promoting a controlled recruitment of gephyrin scaffold. The authors of this study did not investigate the molecular mechanism responsible for collybistin influence on Cdk5 activity. Since Cdk5-dependent phosphorylation of gephyrin is controlled by collybistin expression level, one possible explanation is that Cdk5 catalytic activity is under the control of collybistin because it interacts with it or because gephyrin, while interacting with collybistin, better exposes the side chain of the amino acid residue undergoing post-translational modification.

ERK-DEPENDENT PHOSPHORYLATION OF GEPHYRIN AT Ser268 AFFECTS CLUSTERS SIZE AND DENSITY

Over the past decade, the ERK/MAPK (extracellular signal-regulated protein kinase/mitogen-activated protein kinase) pathway has been implicated in many forms of synaptic plasticity at glutamatergic synapses, including NMDA-dependent and independent forms of LTP. ERK1/2 activity enhances AMPA receptor functional properties by affecting their trafficking, by promoting the structural remodeling of activated spines as well as local protein synthesis (Thomas and Huganir, 2004). At GABAergic synapses ERK1, and to a lesser extent ERK2, were shown to be responsible for gephyrin phosphorylation at a serine residue located in close proximity to the previously recognized target of GSK-3β activity, namely serine 268 (Ser268). This residue attracted attention also because it is not phosphorylated in the C3-gephyrin splice variant, the isoform mainly expressed in non-neuronal cells (Ramming et al., 2000), and this suggests a selective biological significance in neurons.

ERK-mediated phosphorylation at this position was shown to specifically affect the size of post-synaptic gephyrin clusters. Interestingly, ERK and GSK-3β–catalyzed phosphorylations at their corresponding positions became to be functionally interconnected, leading to a coordinated regulation of cluster size

and density paralleled by corresponding changes in amplitude and frequency of GABAergic mIPSCs (Tyagarajan et al., 2013). In other words by inhibiting ERK activity, both cluster density and size were affected, suggesting that ERK exerts a control over GSK-3β activity.l. While the precise dynamics of these events is still unknown it is worth noting that both sites are embedded in a gephyrin domain that contains phosphorylation residues, including putative targets of the prolyl-isomerase Pin1 activity (see below), which render the scenario more complex. Moreover, Ser268 was found acetylated (together with additional nine residues). Even though the functional significance of this type of post-translational modification is unknown, Tyagarajan et al. (2013) hypothesized that acetylation may prevent unwanted phosphorylation by ERK and subsequent down-regulation of GABAergic transmission. Interestingly, ERK activity enhances the strength of glutamateric transmission while decreasing GABAergic transmission, leading to a shift of the E/I balance toward excitation. Therefore, dephosphorylation at Ser268 and/or its acetylation may represent plausible mechanisms to counteract the action of ERK at inhibitory synapses.

Though several issues still remain to be solved, ERK-mediated phosphorylation regulates cluster size *via* calpain activity, as previously demonstrated for GSK-3β–dependent regulation of cluster density. It is interesting to note that application of a broad spectrum phosphatase inhibitor to cultured hippocampal neurons was able to promote the reduction in size of gephyrin clusters, further supporting the functional role of phosphorylation in calpain-dependent gephyrin degradation (Bausen et al., 2010).

Pin1: A NEW PLAYER IN THE ORGANIZATION OF INHIBITORY POST-SYNAPTIC SPECIALIZATIONS

Protein phosphorylation on serine and threonine residues preceding a proline (the so-called proline-directed phosphorylation) has been shown to regulate cell signaling through conformational changes that are not simply due to the phosphorylation event *per se*. Peptidyl-prolyl isomerization of phosphorylated Ser/Thr-Pro sites represents the molecular mechanism utilized by Pro-directed phosphorylation to switch a target substrate between two different functional conformations. The existence of the mechanism relies on the unique stereochemistry of proline residues that within native polypeptides can adopt both *cis* and *trans* conformations. *Cis*-to-*trans* and *trans*-to-*cis* isomerization occur spontaneously but at very low rate: the speed of this event being further reduced upon serine or threonine phosphorylation (Yaffe et al., 1997). These conversions are greatly accelerated by ubiquitous enzymes named peptidyl-prolyl *cis-trans* isomerases (PPIases) or rotamase (Fanghänel and Fischer, 2004). These are divided into 4 families that are unrelated in their primary sequences and three-dimensional structures even though they catalyze the same reaction: cyclophilins (Cyps), FK506-binding proteins (FK506s), parvulins and the PP2A phosphatase activator (PTPA; Jordens et al., 2006). Pin1 and its homologs belong to the parvulin subfamily of PPIase and are the only known enzymes able to isomerise phosphorylated Ser/Thr-Pro sites that become resistant to the catalytic action of conventional prolyl-isomerases (Yaffe et al., 1997). This feature makes the action of Pin1 relevant in the modulation of signaling events, taking into account that Pro-directed kinases and phosphatases are conformation-specific and act only on the *trans* conformation (Weiwad et al., 2000; Zhou et al., 2000).

Pin1 was initially discovered by its ability to interact with the fungal mitotic kinase NIMA (Never In Mitosis A), pointing to an exclusive role for Pin1 in mitosis (Lu et al., 1996). The rapid identification of novel Pin1 substrates has clearly unveiled that this enzyme exerts control over a plethora of cellular processes not only in actively dividing cells but also in fully differentiated cells like post-mitotic neurons. Up to now the best characterized neuronal Pin1 substrates are represented by cytoskeletal proteins such as *tau*, amyloid-β-protein precursor, α-synuclein, and neurofilaments since aberrant interactions with these have implications for the development of neurodegenerative disorders such as Alzheimer disease (Lee et al., 2011), Parkinson disease and amyotrophic lateral sclerosis (Rudrabhatla and Pant, 2010). The involvement of Pin1 in physiological apoptotic events required for the proper development of the nervous system has been also identified (Becker and Bonni, 2006) as well as its contribution for long-lasting forms of synaptic plasticity at excitatory synapses (Westmark et al., 2010).

Gephyrin was identified as a novel target of post-phosphorylation prolyl-isomerization long before its identification as target of Ras/MAPK and PI3K/Akt signaling cascades (Zita et al., 2007). Based on a naïve approach, by inspecting gephyrin amino acid sequence, it was possible to identify 10 putative Pin1 consensus motifs mostly concentrated in the C-domain of gephyrin (**Figure 2**). In particular, while two clusters of three consensus sites were found to be localized within the C-domain, two additional couple of epitopes were located close to the C-terminus of the G-domain and close to the N-terminus of the E-domain, respectively. The C-domain's cluster encompassing the proline-rich region of gephyrin and containing serine 188, 194 and 200, was shown to be responsible for Pin1 recruitment, thus allowing Pin1-driven conformational changes of gephyrin substrate. Functionally, such structural remodeling of gephyrin molecule was shown to affect its binding affinity for the β subunit of the GlyR without affecting its oligomerization properties. In agreement with these findings, hippocampal neurons derived from Pin1 knockout mice demonstrated a loss in the number of GlyR immunoreactive puncta which were mirrored by a concomitant reduction in the amplitude of glycine-evoked currents. These data demonstrated for the first time that post-phosphorylation regulatory mechanisms can affect gephyrin-dependent clustering of inhibitory receptors, rendering it a potential mechanism involved in remodeling the post-synaptic device to sustain synaptic plasticity.

Is Pin1 also involved in GABAergic synaptic signaling? As already mentioned, gephyrin contribution to $GABA_AR$ dynamics requires the coordinated activity of several other associated proteins whose identification and functional characterization has just started to be addressed. At least two key molecules have emerged to play an essential role in regulating gephyrin accumulation at postsynapses, namely NL2 and collybistin (Poulopoulos et al., 2009). These molecules both possess in their sequences

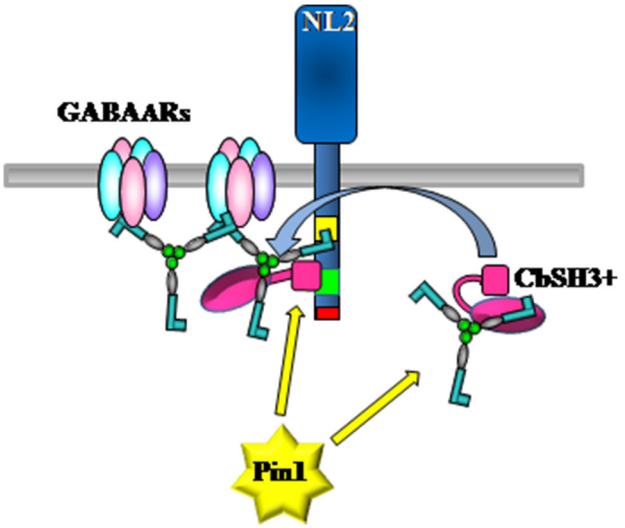

FIGURE 3 | Model of collybistin-driven recruitment of gephyrin by NL2 at GABAergic postsynapses. Pin1 may affect gephyrin/collybistin as well as gephyrin/NL2 interactions leading to an increase or decrease in gephyrin deposition at post-synaptic sites. The cytoplasmic domain of NL2 contains a gephyrin binding domain (yellow), a putative CBD (green) and a C-terminal PDZ binding domain (red).

putative Pin1 consensus motifs, raising the intriguing possibility that post-phosphorylation prolyl-isomerization regulates their reciprocal interaction leading to changes in gephyrin dynamics at synaptic sites (**Figure 3**). Based on this notion, it will be interesting to characterize whether alanine mutagenesis of specific Pin1 consensus sites, in particular the one located within the domains actively engaged in the interaction, would alter (enhancing or weakening) their binding affinity. In addition, it has been demonstrated that, to interact with NL2, gephyrin utilizes a region encompassing the whole C-terminal E-domain linked to a portion of the central region (amino acid 286-736). Two Pin1 consensus sites are present within this gephyrin portion, namely Ser319 and Thr337. As described above, mass spectrometry analysis performed on gephyrin immunoprecipitated from whole rat brain lysates showed that at least Ser319 is phosphorylated *in vivo* (Tyagarajan et al., 2013), making it able to modulate gephyrin/NL2 interaction. In addition, Ser319-Pro is located at the C-terminus of a short amino acid sequence identified as the collybistin binding domain (CBD) on gephyrin (Harvey et al., 2004). Interestingly, the CBD also contains two crucial residues for the interaction with $GABA_A$Rs α1, α2, and α3 subunits, namely Asp327 and Phe330 (Kim et al., 2006; Maric et al., 2011; Tretter et al., 2011). Therefore, a conformational change at this position would influence collybistin recruitment, thus affecting the efficiency of gephyrin synaptic targeting, and perhaps the ability of gephyrin to immobilize $GABA_A$Rs. Pin1 come into play once proline-directed phophorylation has occurred. This molecular switch is therefore positioned downstream the signaling cascades that orchestrate the precise phosphorylation patterns on their corresponding target molecules, thus being able to tune GABAergic transmission.

CONCLUDING REMARKS

The different roles played by the scaffolding molecule gephyrin at GABAergic synapses are still not completely understood. Gephyrin builds a stable scaffold underneath the synaptic plasma membrane to guarantee, over time, the appropriate number of $GABA_A$Rs being juxtaposed to pre-synaptic releasing sites. Despite its overall stability, the gephyrin scaffold must ensure rapid changes in its composition to sustain several forms of synaptic plasticity. One mechanism promoting dynamic changes at inhibitory PSD is represented by post-translational modifications, and in particular by reversible phosphorylation of several key components of the PSDs. The fact that phosphorylation plays a key role in regulating synapse re-arrangement is not new, being extensively characterized at the level of neurotransmitter receptors. The novelty consists in having identified new signaling pathways able to affect synaptic strength by acting on the scaffolding molecule itself *via* alterations of its clustering properties. We are still at the beginning of this new challenge but the data obtained so far disclose a complex scenario. Several serine and threonine residues were found phosphorylated on gephyrin isolated from mouse and rat brains, thus indicating that multiple pathways converge on gephyrin, modifying residues that are very close to each other and possibly functionally interconnected. Interestingly some of the phosphorylated sites were also found acetylated *in vivo*, raising the possibility that acetylation exerts and additional level of control by directly modulating gephyrin protein-protein interaction or by competing with specific phosphorylation targets.

Unveiling the hierarchy of each phosphorylation event, their cross-talks and their respective contribution to the functional organization of GABAergic synapses will require not only the identification of all kinases and phosphatases involved, but also an accurate analysis of their impact on various gephyrin activities, and in particular on $GABA_A$Rs trafficking and synaptic localization.

ACKNOWLEDGMENTS

This work was supported by a grant from Ministero Istruzione Universita' e Ricerca (MIUR, 2011) and from Telethon Foundation (GGP11043) to EC. The authors are grateful to Prof. John Nicholls for carefully reading the manuscript. They wish to thank the colleagues who contributed to some of the original work reported in this review and all members of the laboratory for useful discussions.

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

Received: 30 January 2014; accepted: 22 March 2014; published online: 09 April 2014.
Citation: Zacchi P, Antonelli R and Cherubini E (2014) Gephyrin phosphorylation in the functional organization and plasticity of GABAergic synapses. Front. Cell. Neurosci. ***8****:103. doi: 10.3389/fncel.2014.00103*
This article was submitted to the journal Frontiers in Cellular Neuroscience.

frontiers in
CELLULAR NEUROSCIENCE

MINI REVIEW ARTICLE
published: 28 March 2014
doi: 10.3389/fncel.2014.00093

Modulating excitation through plasticity at inhibitory synapses

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Learning is believed to depend on lasting changes in synaptic efficacy such as long-term potentiation and long-term depression. As a result, a profusion of studies has tried to elucidate the mechanisms underlying these forms of plasticity. Traditionally, experience-dependent changes at excitatory synapses were assumed to underlie learning and memory formation. However, with the relatively more recent investigation of inhibitory transmission, it had become evident that inhibitory synapses are not only plastic, but also provide an additional way to modulate excitatory transmission and the induction of plasticity at excitatory synapses. Thanks to recent technological advances, progress has been made in understanding synaptic transmission and plasticity from particular interneuron subtypes. In this review article, we will describe various forms of synaptic plasticity that have been ascribed to two fairly well characterized populations of interneurons in the hippocampus, those expressing cholecystokinin (CCK) and parvalbumin (PV). We will discuss the resulting changes in the strength and plasticity of excitatory transmission that occur in the local circuit as a result of the modulation of inhibitory transmission. We will focus on the hippocampus because this region has a relatively well-understood circuitry, numerous forms of activity-dependent plasticity and a multitude of identified interneuron subclasses.

Keywords: inhibition, plasticity, PV+, CCK+, hippocampus

DIFFERENT ROLES FOR DIFFERENT INTERNEURONS

It is commonly assumed that changes in inhibitory transmission will have consequences on synaptic plasticity at excitatory synapses. It has been known for over 30 years that pharmacological blockade of γ-Aminobutyric acid (GABA) receptors facilitates the induction of long-term potentiation (LTP) at excitatory synapses (eLTP), likely by increasing Ca^{2+} influx in the postsynaptic cell during the induction protocol (Wigstrom and Gustafsson, 1983). In addition, decreasing inhibition through long-term depression (LTD) at inhibitory synapses (iLTD) can also mediate a dis-inhibitory potentiation of excitatory drive (Ormond and Woodin, 2009).

Interneurons are classified according to several factors including axonal and dendritic connectivity, electrophysiological properties and expression of molecular markers. Based on these criteria, the hippocampus is one of the structures with the largest interneuron diversity (reviewed by Somogyi and Klausberger, 2005). In this mini-review, we will describe several forms of plasticity that have been ascribed to specific interneuron populations and discuss the resulting changes in the strength and plasticity at excitatory transmission. We will focus on hippocampal interneurons expressing cholecystokinin (CCK) and parvalbumin (PV). These two populations of interneurons are relatively well characterized in multiple brain areas (Freund and Katona, 2007; Armstrong and Soltesz, 2012) and recent studies have benefitted from genetic tools allowing their identification and modulation in hippocampal slices and *in vivo*. Our focus is restricted to studies performed in the hippocampus because the well-characterized circuitry has allowed for insight into how the numerous forms of plasticity expressed in inhibitory cells alters excitation and modulates network properties.

CCK+ interneurons are considered to be highly plastic, as several neurotransmitters and neuromodulators have been revealed to alter synaptic transmission from these cells. PV+ interneurons, on the other hand, have been considered to be much more static, acting to control the firing frequency and timing of pyramidal cells. However, there is recent evidence that synaptic transmission from and onto PV+ interneurons can be plastic. We will briefly describe how GABA release from CCK+ and PV+ interneurons can be modulated, and discuss the consequences of these modulations on the excitatory plasticity and overall network function in the hippocampus (**Figures 1, 2**).

MODULATION OF GABA RELEASE BY CCK+ AND PV+ INTERNEURONS

The kinetics of GABA vesicle fusion has been found to differ between CCK+ and PV+ interneurons. Axon terminals of CCK+ cells express N-type Ca^{2+} channels. These Ca^{2+} channels are loosely coupled to the Ca^{2+} sensor involved in vesicle fusion, thereby resulting in significant jittering and asynchronous release of GABA (Hefft and Jonas, 2005). Furthermore, different types of CCK+ interneurons, including basket cells, bistratified cells and

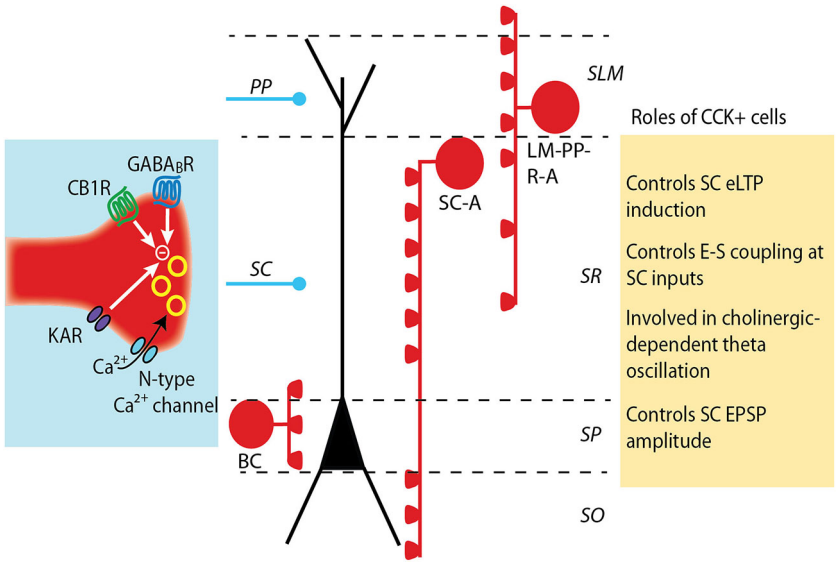

FIGURE 1 | Modulation and roles of GABA release from CCK+ interneurons. CCK+ interneurons target pyramidal cell soma (basket cell, BS) or dendrites (Schaffer collateral-associated cell, SC-A and lacunosum moleculare-radiatum-perforant path-associated cell, LM-R-PP-A) (see Somogyi and Klausberger, 2005). The release of GABA from CCK+ cell terminals is mediated by N-type calcium channels, which provide a loose coupling between calcium influx and exocytosis and partially underlie the asynchronous release of GABA by these cells. GABA release is negatively controlled by the activation of several receptors: CB1 cannabinoid receptors, $GABA_B$ receptors and kainate receptors. The decrease in GABA release differently impacts excitatory synapses depending on which subset of CCK+ interneuron synapses are depressed. A decrease in dendritic-targeting CCK+ synapse facilitates LTP induction at SC-CA1 synapses and increases the ability of an excitatory post synaptic potential (EPSP) to evoke an action potential (E-S coupling). When GABA release at somatic-targeting CCK+ synapses is depressed, a large increase in the amplitude of the SC EPSPs is observed, but distal perforant path (PP) EPSPs are unaltered.

trilaminar cells show asynchronous release (Daw et al., 2009). In contrast, PV+ interneuron axon terminals express P/Q-type Ca^{2+} channels, which are more tightly coupled to vesicle fusion because of their location in the active zone. As a consequence, PV+ interneurons have more synchronous release of GABA (Hefft and Jonas, 2005).

CCK itself, which is co-released with GABA, can modulate GABA release by both CCK+ and PV+ neurons (Földy et al., 2007). In CCK+ cells, CCK release activates CCK2 receptors on pyramidal cells, resulting in retrograde endocannabinoid release and pre-synaptic activation of cannabinoid type 1 receptors (CB1R) and reducing GABA release (Földy et al., 2007; Karson et al., 2008). In contrast, activation of CCK2 receptors on PV+ basket cells results in the activation of a pertussis-toxin sensitive G-protein (Gi/o) coupled pathway that results in intracellular calcium release, transient receptor potential (TRP) channel activation and membrane depolarization (Lee et al., 2011). This membrane depolarization results in increased GABA release. Interestingly, PV+ bi-stratified cells showed no response to CCK, indicating that this modulation is specific to somatic inhibition and may be an important complementary component to the CCK+ cell modulation by CCK.

GABA release by CCK+ cells is uniquely altered by several modulators. For instance, the synchronous release of GABA can be decreased by presynaptic kainate receptors (Daw et al., 2010). In addition, the $GABA_B$ receptor is detected in CCK+ cells but not in PV+ interneurons (Sloviter et al., 1999) and there is experimental evidence suggesting that activation of these receptors powerfully decreases GABA release from CCK+ cells (Neu et al., 2007). Furthermore, CCK+ cells are likely the only class of interneurons expressing CB1R (Marsicano and Lutz, 1999; Takács et al., 2014).

The exclusive modulation of one type of interneuron can have interesting functional consequences. For example, in fast-spiking PV+ basket cells, mu-opioid receptor activation hyperpolarizes the membrane and depresses GABA release while nearby CCK+ basket cells are unaffected by the mu-opioid receptor activation but are uniquely modulated by cannabinoid application (Glickfeld et al., 2008). Likewise, a comparison of the action of acetylcholine on different soma-targeting PV+ and CCK+ basket cells revealed that GABA release was diminished by M2-type muscarinic receptor activation uniquely in PV+ cells whereas CCK+ cell transmission was inhibited via cannabinoid signaling (Szabó et al., 2010). Thus, even though these interneuron classes receive similar inputs and have similar axonal arbors, their properties endow them with very different frequency tuning properties and are likely active at different times (Glickfeld and Scanziani, 2006). Furthermore, the distinct modulation of these different perisomatic interneurons can act to shift pyramidal cells into different modes of integration.

CONSEQUENCES OF CCK+ INTERNEURON PLASTICITY ON EXCITATORY CELL TRANSMISSION

The CB1R is one of the most highly expressed G protein-coupled receptors in the nervous system (Herkenham et al., 1990). These

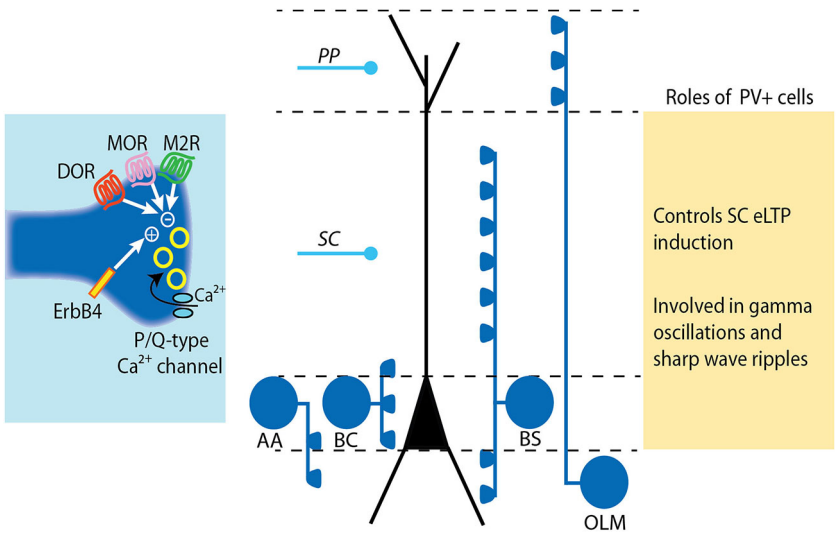

FIGURE 2 | Modulation and roles of GABA release from PV+ interneurons. PV+ interneurons target either the soma (basket cell, BS), the axon (axo-axonic cell, AA) or the dendrites (bistratified cell, BS and oriens-lacunosum moleculare cell, OLM) of pyramidal cells (see Somogyi and Klausberger, 2005). The release of GABA from PV+ cell terminals is mediated by P/Q-type calcium channels. The tight coupling between calcium influx and exocytosis machinery results in precisely timed vesicle release. The release of GABA at PV+ cell synapses is negatively controlled by diverse receptors including mu- and delta-opioid receptors (MOR and DOR) and muscarinic M2 receptors. Conversely, GABA release from PV cells is increased by activation of the Neuregulin 1 receptor ErbB4. LTP induction at SC-CA1 synapses is impaired following ErB4 activation in PV+ cells due to increased pre-synaptic GABA release by PV+ cells.

receptors are involved in the action of endogenous cannabinoids (eCBs), which are synthetized from membrane lipid precursors by the postsynaptic cell and act as retrograde messengers to depress transmitter release from presynaptic terminals (for a general review, see Chevaleyre et al., 2006). All of the CB1-dependant plasticity discussed below are known to occur in CCK+ interneurons; however, it should be noted that not all CCK+ interneurons express CB1 receptors.

In the hippocampus, eCBs are involved in two forms of synaptic plasticity. When transiently released, for instance by depolarization of the postsynaptic cell, they mediate a short-term (∼1 min) depression of GABA release, a phenomenon called depolarization-induced suppression of inhibition (DSI). This phenomenon was initially described more than 20 years ago in the cerebellum (Llano et al., 1991) and hippocampus (Pitler and Alger, 1992). The retrograde action of eCBs was attributed by Wilson and Nicoll (2001). The fast onset of DSI and the lack of sensitivity of tetrodotoxin (TTX) -resistant miniature IPSCs to DSI (Alger et al., 1996) are in agreement with a direct block of N-type Ca^{2+} channels by β/γ subunits of the G protein, an effect that was initially demonstrated in expression systems (Mackie and Hille, 1992).

When a more sustained release of eCB is evoked, for instance following activation of group I metabotropic glutamate receptor (mGluR-I), eCBs can mediate a long-term depression of inhibitory transmission. Several minutes of CB1R activation are needed for a lasting depression to be induced. This more sustained CB1R activation probably allows for significant changes in second messenger and phosphorylation levels of downstream target molecules. Consistently, protein kinase A (PKA) activity and the active zone proteins RIM1α and Rab3b are needed for iLTD induction (Chevaleyre et al., 2007; Tsetsenis et al., 2011), indicating that iLTD results in a change on the release machinery.

Because of the specific expression of CB1R in CCK+ cells, eCB-mediated plasticity initially offered a useful tool to dissect out the role of CCK+ cells in controlling excitatory transmission. Several studies reported that the decrease in GABA release from CCK+ cells could facilitate LTP induction at the Schaffer collateral (SC) -CA1 excitatory synapse. Not surprisingly, the time course of the facilitation follows the time course of the eCB-mediated plasticity. For instance, it was initially described that the dis-inhibition occurring during DSI provides a transient facilitation of LTP induction at excitatory synapses (Carlson et al., 2002). In contrast, induction of iLTD by eCB provides a long-lasting facilitation on the induction of eLTP (Chevaleyre and Castillo, 2004; Zhu and Lovinger, 2007). The spatial localization of the facilitation depends on the induction protocol used to evoke eCB release. DSI is a single-cell phenomenon, thus eLTP facilitation will only occur onto the cell expressing DSI. However, DSI targets multiple CB1R-sensitive inhibitory synapses along the somato-dendritic compartment, and will likely facilitate LTP induction at excitatory inputs targeting different locations of the apical dendrite. In contrast, iLTD can be evoked by a very localized activation of the Schaffer collaterals. The activation of mGluR-I onto pyramidal neurons triggers eCB release that hetero-synaptically decreases GABA release from nearby inhibitory terminals (Chevaleyre and Castillo, 2003). Because iLTD is spatially restricted to the region surrounding the stimulated excitatory fibers, eLTP facilitation is limited to the nearby dendritic region (Chevaleyre and Castillo, 2004). However, it was recently reported that iLTD can

be evoked with repetitive postsynaptic firing, indiscriminately affecting somatic and dentritic inhibitory inputs (Younts et al., 2013). Therefore, it is expected that facilitation of eLTP will not be spatially restricted following this mode of induction.

Independently of the facilitation of eLTP described above, the decrease in GABA release from CCK+ cells can also increase the ability of an excitatory post synaptic potential (EPSP) to evoke an action potential (E-S coupling) and directly increase the size of the EPSP at the SC-CA1 synapse. The first effect was observed after inducing iLTD with synaptic activity of the SC inputs or with repetitive postsynaptic depolarization. Action potential firing was extracellularly monitored (Chevaleyre and Castillo, 2003) or recorded in individual pyramidal cells (Younts et al., 2013), and was increased with both iLTD inducing protocols. The second effect, i.e., a direct increase in the amplitude of SC EPSP, was reported recently by two studies using a paired stimulation between proximal (SC) and distal perforant path (PP) excitatory inputs, termed input-timing dependent plasticity (ITDP; Dudman et al., 2007). The initial study showed that the pairing protocol induced a potentiation of SC-EPSPs, and that this potentiation is dependent on eCB-mediated LTD at inhibitory synapses (Xu et al., 2012). The dependence on CB1R strongly suggests that the interneurons expressing iLTD were CCK+ cells. This idea was formally demonstrated in a second thorough and elegant study using multiple techniques to better elucidate the phenomenon (Basu et al., 2013). The authors showed that transmission from CCK+ interneurons is depressed following the ITDP protocol. In addition, this depression concerns perisomatic CCK+ terminals and is mediated by eCB release during the ITDP protocol. Finally, they showed that most of the increase in EPSP amplitude following the ITDP protocol is the result of the eCB-mediated iLTD at CCK+ terminals. These studies convincingly show that CCK+ interneurons targeting the soma of pyramidal neurons are playing an important role in controlling the strength of SC inputs. These data therefore suggest that CCK+ interneurons should contribute significantly to the feed-forward (FF) inhibition evoked by SC stimulation. Indeed, using optogenetics to silence CCK+ interneurons, the authors show that CCK+ cells mediate a major proportion of the FF inhibition elicited by SC stimulation, and that silencing transmission from CCK+ cells induced a large increase in SC-mediated EPSPs.

Altogether, these studies reveal a dual role of CCK+ interneurons in the control of excitatory transmission and plasticity. While a decrease in GABA release from dendritic-targeting CCK+ cells can facilitate LTP induction at SC-CA1 excitatory synapses, a decrease in GABA release from somatic-targeting CCK+ basket cells will directly increase the amplitude of the EPSPs. These studies highlight the importance of determining the subclass of interneuron by using a combination of protein markers, physiological properties and dendritic and axonal arborizations.

CONSEQUENCES OF PV+ INTERNEURON MODULATION ON EXCITATORY CELL TRANSMISSION

Excitatory synapses onto interneurons are known to express either LTP or LTD via activation of calcium-permeable glutamate receptors (Kullmann and Lamsa, 2007). Cell-type specific rules have been identified in a study examining five common interneuron subtypes, as defined by axonal projections and molecular expression profiles (Nissen et al., 2010). In this work, the authors found that excitatory synapses express LTP onto PV+ basket cells and LTD onto bistratified cells. Both of these phenomena were independent of N-Methyl-D-aspartate (NMDA) receptor activation and potentially act to shift the inhibition on excitatory cells from the dendrites to the soma. A closer examination of the FF and feedback (FB) excitatory inputs onto PV+ basket cells found that NMDA receptors are only found at synapses with FB afferents, leading to a narrower frequency tuning of LTP at these inputs than at FF inputs (Le Roux et al., 2013). Given the importance of FF inhibition to ensure the temporal fidelity of pyramidal cell firing (Pouille and Scanziani, 2001) and the very tight time-lock of basket cell interneurons and pyramidal cells during sharp wave ripples (Klausberger and Somogyi, 2008), it is possible the different properties of LTP at FF and FB synapses is permitting PV+ cells to modulate their activity in accordance with excitation.

PV+ basket cells in area CA1 have recently been shown to undergo a long-term increase in excitability in response to brief high frequency stimulation of SC inputs (Campanac et al., 2013). It was elegantly shown that this enhanced FF inhibition in area CA1 was due to an increase in the inherent excitability of PV+ cells resulting from activation of mGluR5 and subsequent down-regulation of D-type potassium current carried by Kv1 channels, termed LTP-IE$_{PV-BC}$. The authors demonstrated that clustered spiking in the γ-range was increased, allowing for the speculation that this plasticity may provide a use-dependent modulation of hippocampal γ-oscillations, or even potentially allow for a modulation of the phase lag of PV+ basket cells during θ-activity.

PV+ interneurons may be playing an interesting role in mediating the ability of CA3 neurons to excite CA2 pyramidal cells. A very strong FF inhibition at the SC-CA2 synapse induces a very large hyperpolarization in CA2 pyramidal neurons and completely prevents SC axons from driving firing in CA2 pyramidal neurons (Chevaleyre and Siegelbaum, 2010; Kohara et al., 2014). Upon closer examination, this inhibition was found to undergo an iLTD in response to 10, θ-burst, and 100 Hz stimulus protocols. Furthermore, this iLTD was mediated entirely by the activation of delta opioid receptors, resulting in a lasting decrease in GABA release. Furthermore, by using optogenetics to elicit an IPSC from PV+ interneuron terminals, it was demonstrated that PV+ interneurons are responsible for this plasticity (Piskorowski and Chevaleyre, 2013). Given that CA2 pyramidal neurons express multiple factors that inhibit post-synaptic LTP at the SC-CA2 synapse (Zhao et al., 2007; Simons et al., 2009; Lee et al., 2010), this pre-synaptic iLTD in PV+ cells may be the major mechanism by which the excitability of the SC-CA2 synapse is modulated.

Changes in PV+ cell plasticity during development is thought to underlie the "critical periods" in cortical development when neural circuits undergo large adaptations in response to the environment (see review by Takesian and Hensch, 2013). Interestingly, there is growing evidence that PV+ cells in adult hippocampal circuits are modulated by similar mechanisms during learning.

This premise is supported by the finding that the trophic factor neuregulin1 (NRG1), which is a critical element in PV+

maturation during development, acts on adult PV+ cells to increase GABA transmission, resulting in a suppression of LTP induction at SC-CA1 synapses (Pitcher et al., 2008; Chen et al., 2010). When the NRG1 receptor, ErbB4, was selectively knocked out from PV+ cells, LTP at SC-CA1 synapses was increased and no longer repressed by NRG1. Interestingly, the PV+ cell specific ErbB4 knockout animals display a deficit in contextual fear conditioning, revealing an important role of PV+ cells in hippocampal learning (Chen et al., 2010). A recent and compelling study by Donato et al. (2013) has shown that PV+ basket cells in area CA3 show a change in activity state following contextual fear conditioning or environmental enrichment, two treatments found to respectively decrease or improve performance of hippocampal-dependent novel object recognition. The intensity of PV staining at axonal terminals was used as an indicator of PV+ cell activity state: high PV levels in the non-plastic state and low PV levels in the highly-plastic state. These observations are consistent with previous reports that pre-synaptic PV levels are able to modulate pre-synaptic calcium levels and GABA release in cerebellar interneurons during development (Collin et al., 2005). Furthermore, manipulations of the perineuronal net, an extracellular matrix that grows and shrinks during developmental "critical periods" and releases NRG1 and other PV+ cell modulators, can reset the PV+ cells to a highly-plastic low PV condition in the adult hippocampus (Donato et al., 2013) indicating that additional factors regulating PV+ cell excitability during development may control plasticity in the adult.

PERSPECTIVE

At the circuit level, a recent study reported that cholinergically-driven θ-oscillations in CA1 involves an inhibitory circuit consisting mainly of CCK+ interneurons (Nagode et al., 2014). This conclusion was based on the observation that cholinergically-driven oscillatory IPSCs were sensitive to cannabinoids and optogenetic silencing of CCK+ cells, but not PV+ cells. Thus, while CCK+ cells may be involved in low frequency oscillations such as θ-rhythm, PV+ cells may play a more prominent role in faster rhythms such as γ and sharp wave ripple oscillations. However, removing inhibition onto PV+ interneurons also affects θ-oscillation *in vivo* in CA1, suggesting a complex interaction between different interneuron types in oscillatory activity (Wulff et al., 2009). With the high diversity of PV+ interneurons in the hippocampus, there is also a very large level of diversity of synaptic plasticity. Recordings performed *in vivo* that take into account the axonal arbors and cell-type specific markers of interneurons, have revealed that each cell type has a potential role in the network activity of the hippocampus during a specific behavior (for example, Klausberger et al., 2003, 2004; Tukker et al., 2007; Lapray et al., 2012). Distinct differences in PV+ cell projection patterns and activities have been found in different hippocampal regions (Tukker et al., 2013). Even with all of this complexity, *in vivo* studies in which transmission from all PV+ cells has been removed reveal very interesting changes in behavior and hippocampal network activity (Korotkova et al., 2010; Murray et al., 2011; Royer et al., 2012). Deciphering how each subclass of interneuron dynamically contributes to network function during learning and disease states is a worthy goal for future work.

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

Received: 31 January 2014; accepted: 13 March 2014; published online: 28 March 2014.
Citation: Chevaleyre V and Piskorowski R (2014) Modulating excitation through plasticity at inhibitory synapses. Front. Cell. Neurosci. ***8****:93. doi: 10.3389/fncel.2014.00093*
This article was submitted to the journal Frontiers in Cellular Neuroscience.